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Cytogeography of *Callisia* section *Cuthbertia* (Commelinaceae)

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Abstract

Determining the distribution of cytotypes across the geographic distribution of polyploid complexes can provide valuable information about the evolution of biodiversity. Here, the phytogeography of cytotypes in *Callisia* section *Cuthbertia* (Small, 1903) Hunt, 1986 is investigated. A total of 436 voucher specimens was georeferenced; 133 new specimens were collected. Based on flow cytometry data, DNA content of all cytotypes in *Callisia* section *Cuthbertia* was estimated. Utilizing chromosome counts and flow cytometric analysis, cytotype distribution maps were generated. Two disjunct groups of populations of diploid *Callisia graminea* (Small, 1903) Tucker, 1989 were discovered; tetraploid *C. graminea* ranges broadly from the coastal plain of North Carolina through central Florida. One hexaploid *C. graminea* individual was recorded in South Carolina, and numerous individuals of hexaploid *C. graminea* were found in central Florida. Diploid *C. ornata* (Small, 1933) Tucker, 1989 occurs in eastern Florida; previously unknown tetraploid and hexaploid populations of *C. ornata* were discovered in western and central Florida, respectively. Diploid *C. rosea* (Ventenat, 1800) Hunt, 1986 occurs in Georgia and the Carolinas, with populations occurring on both sides of the Fall Line. The cytotype and species distributions in *Callisia* are complex, and these results provide hypotheses, to be tested with morphological and molecular data, about the origins of the polyploid cytotypes.

Keywords

chromosome counts, cytotypes, endemic, Florida scrub vegetation, flow cytometry, genome size, polyploidy, sandhill vegetation, Southeastern United States

Introduction

Polyploidy (whole-genome duplication) is a speciation mechanism that is a major evolutionary force; in fact, all angiosperms have undergone at least one ancient polyploidy event (Jiao et al. 2011, *Amborella* Genome Project 2013), and polyploidy has been a key driver of angiosperm diversity (De Bodt et al. 2005, Soltis et al. 2009, Soltis and Soltis 2009, Soltis and Soltis 2016, Tank et al. 2015).

Polyploids are classified in two major categories: allopolyploids and autopolyploids. Allopolyploids are by far the more studied form and arise via hybridization between species, whereas autopolyploids originate from the multiplication of genomes within a single species. An autopolyploid is frequently considered as a cytotype within a species along with its diploid progenitor, as in *Galax urceolata* (Poiret, 1804) Brummitt, 1972 (Baldwin 1941, Stebbins 1950), *Chamerion angustifolium* (Linnaeus, 1753) Holub, 1972 (Mosquin 1967), *Heuchera grossulariifolia* Rydberg, 1900 (Wolf et al. 1990), and *Vaccinium corymbosum* Linnaeus, 1753 (Camp 1945, Krebs and Hancock 1989). However, autotetraploids are occasionally recognized as species distinct from their diploid parent, such as *Zea perennis* (Hitchcock, 1922) Reeves & Mangelsdorf, 1942 (Iltis et al. 1979, Tiffin and Gaut 2001) and *Tolmiea menziesii* Torrey & Gray, 1840 (Judd et al. 2007). Lumping diploid progenitors with their multiple derivative cytotypes into a single species may mask evolutionary lineages and grossly underestimate biodiversity (Soltis et al. 2007).

To gain a better assessment of biodiversity and to guide conservation efforts for species of interest, data on both evolutionary and life-history characteristics are needed. *Callisia* section *Cuthbertia* (Commelinaceae) from the southeastern U.S.A. comprises a polyploid complex, with species of conservation concern, but the extent of polyploidy and the geographic distribution of cytotype diversity are unknown.

Callisia Loefling,1758 is one of 39 genera in subfamily Commelinoideae (Burns et al. 2011) and is placed in tribe Tradescantieae subtribe Tradescantiinae. *Callisia* comprises approximately 23 species in six sections (*Hadrodemas* (Moore, 1963) Hunt, 1986, *Cuthbertia* (Small, 1903) Hunt, 1986, *Lauia* Hunt, 1986, *Brachyphylla* Hunt, 1986, *Leptocallisia* Bentham & Hooker, 1883, and *Callisia*) (Hunt 1986, Tucker 1989). Of these sections, *Cuthbertia* is endemic to the U.S.A., and *Brachyphylla*, *Leptocallisia*, and *Callisia* also have members that occur in the U.S.A. (Tucker 1989). The remaining two sections (*Lauia* and *Hadrodemas*) occur in Central America, South America, and the Caribbean. In recent phylogenetic analyses, *Callisia* is not monophyletic (Bergamo 2003, Burns et al. 2011), although, significantly, section *Cuthbertia* is monophyletic in all analyses (Bergamo 2003, Burns et al. 2011, Hertweck and Pires 2014).

Callisia section *Cuthbertia* consists of three morphologically distinct species (*C. graminea*, *C. ornata*, and *C. rosea*) that are endemic to the southeastern U.S.A. and have a base chromosome number of $x = 6$ (Giles 1942, 1943). *Callisia graminea* (Small, 1903) Tucker, 1989, the grassleaf roseling, occurs from the southern border of Virginia through central Florida. Giles (1942, 1943) reported three ploidal levels ($2x$, $4x$, and $6x$) for this species and encountered a single triploid individual in Hoke County, NC. Based on cytological criteria, the tetraploid was interpreted as an autopolyploid derivative of diploid *C. graminea* (Giles 1942, 1943). The nature of polyploidy in hexa-

ploid *C. graminea* is not clear. Within *C. graminea*, two forms have been described: *C. graminea* forma *graminea* has pink flowers with anthocyanin pigments, and *C. graminea* forma *leucantha* (Lakela, 1972) Tucker, 1989 has white flowers and was described from two diploid cuttings (Lakela 1972). *Callisia ornata* (Small, 1903) Tucker, 1989 (Florida scrub roseling), a diploid (Giles unpublished), is endemic to central to southern Florida. *Callisia rosea* (Ventenat, 1800) Hunt, 1986 (Piedmont roseling) is a diploid (Anderson and Sax 1936), with a distribution from North Carolina to Georgia.

Although earlier studies (e.g., Giles 1942, 1943) provided the general pattern of species distributions and cytotypic diversity, the extent of cytotypic variation within and among species has not been examined in detail. Additional sampling of both populations and species is required to understand the extent and distribution of cytological variation in this clade. In this study, numerous new field collections were made, and known populations of *Callisia* section *Cuthbertia* were revisited; with the use of both traditional chromosome counts and flow cytometry, the ploidy of samples spanning the entire range of *Callisia* section *Cuthbertia* was investigated. Distribution maps of cytotypes and species were generated based on the cytological data obtained here, enabling future studies of phylogeny and polyploid origins in *Callisia* section *Cuthbertia*.

Materials and methods

Georeferencing

To obtain locality data for *Callisia graminea*, *C. ornata*, and *C. rosea*, voucher specimens were examined from the following herbaria: GA, USCH, NCU, DUKE, US, AAH, FLAS, FSU, VSC, and SFU (codes follow Thiers 2016). The locality of each specimen was georeferenced by manually incorporating the label data into the web applications ACME mapper 2.1 (Poskanzer 2001) and/or GEOLocate (Rios and Bart 2010). Additional localities were obtained from the Master's Thesis of A. Kelly (1991) and personal communications with members of the Florida Native Plant Society and photographers from Flickr.com. In all, 436 specimens were georeferenced from herbarium specimens and observation records. (See supplementary file 1: Table 1 for georeferenced data points.) The data points were used to produce a distribution map using ArcGIS 10.4 (ESRI 2016) and to locate known populations and contact zones of all three species and their cytotypes.

Collecting of specimens

The georeferenced data were used to relocate populations within the southeastern U.S.A.; additional localities were discovered by exploring similar habitats in protected areas and on private land. Collections on private land were made with permission of the land owners. Based on the georeferenced data, permits were obtained to collect in state parks, state forests, national parks, and protected areas of The Nature Conservancy and the U.S. Fish and Wildlife Service in Florida, Georgia, South Carolina, North Carolina, and Virginia (Table 1).

Table 1. Populations used in this study. Geographic location, ploidy, number of plants of each ploidy, total number of analyzed individuals, and voucher information for 133 populations of *Callisia graminea* (G), *C. ornata* (O), and *C. rosea* (R) from the southeastern United States. * indicates a new locality with voucher specimen.

				Geographic coordinates		Ploidy / Number of plants				
Population	Locality	State	County	Latitude	Longitude	2*x*	4*x*	6*x*	*N*	Voucher no.
Callisia graminea (Small) G. Tucker										
G-1*	Gainesville Regional Airport	FL	Alachua	29°42.01'N	082°15.72'W		1		3	307
G-2	Jct. Tower Rd. and SW 8 Ave	FL	Alachua	29°38.63'N	082°25.24'W		1		4	223
G-3	Morningside Nature Center	FL	Alachua	29°39.56'N	082°16.45'W		1		1	234
G-4	Jct. Hwy 200 and CR. 491	FL	Citrus	28°58.51'N	082°21.84'W		1		2	229
G-5*	Along Rod Rd.	FL	Clay	30°01.52'N	081°51.95'W		1		1	225
G-6	Golden Branch Head State Park	FL	Clay	29°50.75'N	081°57.04'W		1		2	309
G-7	Silver Sand Lake Rd.	FL	Clay	29°47.49'N	081°58.32'W		1		4	311
G-8*	Tate Hell State Forest along New River	FL	Franklin	29°52.42'N	084°41.79'W		1		4	306
G-9*	Richloam State Forest/Dark Stretch Rd.	FL	Hernando	28°29.10'N	082°08.87'W			1	6	349
G-10*	Edwards Rd., Lady Lake	FL	Lake	28°54.12'N	081°53.40'W			1	3	235
G-11*	Lake Griffin State Park	FL	Lake	28°52.31'N	081°53.41'W			1	3	236
G-12*	Seminole State Forest along Co. Rd. 42	FL	Lake	29°00.82'N	081°31.05'W		1	1	3	345
G-13*	Seminole State Forest	FL	Lake	28°49.31'N	081°28.01'W		1		1	362
G-14*	Lake Norris Rd.	FL	Lake	28°54.89'N	081°32.41'W		1		1	363
G-15*	ATV trail at Ocala National Forest	FL	Marion	29°21.76'N	081°44.21'W		1		1	230
G-16	Silver River State Park	FL	Marion	29°12.15'N	082°02.77'W		1		4	348
G-17*	Along Mason Rd.	FL	Putnam	29°42.50'N	082°00.77'W		1		2	224
G-18*	Ordway Biological Center H1 & H2 area	FL	Putnam	29°41.70'N	081°57.87'W		1		2	302
G-19*	Etoniah Creek State Forest	FL	Putnam	29°46.43'N	081°51.91'W		1		3	308
G-20	Dunns Creek State Park entrance Sisco Rd.	FL	Putnam	29°31.84'N	081°35.34'W		1		4	310
G-21*	Welaka State Forest	FL	Putnam	29°28.24'N	081°39.37'W		1		2	360a
G-22	Along State Rd. 46	GA	Bulloch	32°20.94'N	081°50.57'W		1		3	242
G-23	Jct. Hwy 185 and Turkey Ridge Dr.	GA	Charlton	30°24.76'N	082°11.70'W		1		2	317

				Geographic coordinates		Ploidy / Number of plants				
Population	Locality	State	County	Latitude	Longitude	2*x*	4*x*	6*x*	*N*	Voucher no.
G-24*	General Coffee State Park	GA	Coffee	31°31.50'N	082°46.33'W		1		1	318
G-25	N. Connector Rd./206 Jct. 135	GA	Coffee	31°32.27'N	082°48.75'W		1		3	319
G-26*	George Smith State Park	GA	Emanuel	32°32.64'N	082°07.32'W		1		6	241
G-27*	Ochicoo Preserve, Halls Bridge Rd.	GA	Emanuel	32°31.73'N	082°27.38'W		1		4	320
G-28	Fort Stewart	GA	Evans	32°06.92'N	081°47.10'W		1		4	243
G-29*	Conway CT./Interstate Parkway	GA	Richmond	33°29.24'N	082°06.12'W		1		1	322
G-30	Fort Gordon	GA	Richmond	33°23.33'N	082°14.56'W					239
G-31*	Singletary Lake State Park	NC	Bladen	34°35.41'N	078°26.87'W		1		3	263
G-32*	Jones Lake State Park	NC	Bladen	34°42.11'N	078°37.22'W		1		3	268
G-33*	Jones Lake State Park	NC	Bladen	34°42.11'N	078°37.22'W		1			269
G-34*	Along NC 242 near Jones Lake State Park	NC	Bladen	34°42.00'N	078°36.35'W		1		2	270
G-35*	Along NC 242 N. of Jones Lake State Park	NC	Bladen	34°45.40'N	078°36.56'W		1		5	271
G-36*	White Lake, along NC 741, Barnes Food Co.	NC	Bladen	34°39.41'N	078°30.17'W		1		5	272
G-37*	Jones Lake State Park. campsite	NC	Bladen	34°40.79'N	078°35.99'W					274
G-38*	Along Burney Rd. underneath powerline	NC	Bladen	34°44.38'N	078°43.68'W		1		4	334
G-39*	River Rd., underneath powerline	NC	Bladen	34°46.18'N	078°47.24'W		1		3	335
G-40	Bay Tree Lake State Park/undeveloped	NC	Bladen	34°40.22'N	078°25.66'W		1		6	261
G-41	Along Hwy 41 close to Bay Tree Lake State Park	NC	Bladen	34°41.21'N	078°25.26'W		1		3	262
G-42	Along Hwy 11 towards Delco under powerline	NC	Bladen	34°24.61'N	078°15.60'W		1		4	266
G-43	Along Jessup Pond	NC	Bladen	34°51.72'N	078°43.76'W					275
G-44	Lake Waccamaw State Park.	NC	Columbus	34°16.73'N	078°27.89'W					267
G-45*	Mack Simmons Rd.	NC	Cumberland	34°54.45'N	078°44.20'W					276
G-46*	Along NC 210, Jct. with Sidney Bullard Rd.	NC	Cumberland	34°58.69'N	078°43.84'W		1		4	278
G-47*	Ft. Bragg/John Mill Rd.	NC	Cumberland	35°10.70'N	079°05.39'W	1			3	341
G-48*	Ft. Bragg/NE. training/Mc Closkey Rd.	NC	Cumberland	35°09.84'N	078°56.97'W	1			3	342
G-49	Cedar Creek Rd., Tatum farm	NC	Cumberland	34°56.32'N	078°44.58'W		1		1	277

				Geographic coordinates		Ploidy / Number of plants				
Population	Locality	State	County	Latitude	Longitude	2*x*	4*x*	6*x*	*N*	Voucher no.
G-50	Along Dunns Rd./NC 301	NC	Cumberland	35°06.42'N	078°46.52'W					279
G-51	Open Area along NC 24	NC	Harnett	35°15.61'N	079°02.47'W	1			3	284
G-52	Along Rockfish Rd.	NC	Hoke	34°59.32'N	079°05.82'W	1			3	286
G-53	In open area along Red Springs Rd.	NC	Hoke	34°52.38'N	079°12.17'W	1			4	287
G-54*	Weymouth Sandhill Nature Preserve	NC	Moore	35°08.95'N	079°22.10'W	1			3	288
G-55	Along Riverview Dr.	NC	Moore	35°11.48'N	079°10.94'W	1			3	285
G-56	Along NC 11/ Hwy 53	NC	Pender	34°29.72'N	078°11.49'W		1		3	264
G-57	Along NC 11/ Hwy 53	NC	Pender	34°29.72'N	078°11.49'W		1		1	265
G-58*	Grey Woods Rd.	NC	Richmond	34°57.52'N	079°38.47'W	1			3	297
G-59*	Sandhills Game Land	NC	Richmond	35°01.83'N	079°36.70'W	1			2	336
G-60*	Sandhills Game Land/442/Ledbetter Rd.	NC	Richmond	35°03.62'N	079°38.09'W	1			3	337
G-61*	Sandhills Game Land	NC	Richmond	34°58.61'N	079°30.42'W	1			2	338
G-62*	Sandhills Game Land SR 1331, 15/501	NC	Richmond	34°58.50'N	079°26.93'W	1			2	339
G-63*	Sandhills Game Land, Aberdeen Rd./Hill Creek Rd.	NC	Richmond	34°59.49'N	079°26.76'W	1			3	340
G-64	Sandhills Game Land along McDonald Church Rd.	NC	Richmond	35°01.24'N	079°37.18'W	1			2	290
G-65	NC Hwy 177	NC	Richmond	34°50.41'N	079°45.54'W		1		1	295
G-66	Along Saint Stevens Church Rd.	NC	Richmond	34°49.82'N	079°50.55'W	1			1	296
G-67	NC 242, 0.3 mi N. of Cumberland Co. line	NC	Sampson	34°53.35'N	078°31.28'W		1		3	273
G-68	Along Spiveys Corner Hwy.	NC	Sampson	35°10.72'N	078°28.65'W	1			2	280
G-69	Edge camp Mackall along Aberdeen Rd.	NC	Scotland	35°00.84'N	079°26.70'W	1			2	289
G-70	Along 1328, Hoffman Rd./Butler Rd.	NC	Scotland	34°59.14'N	079°31.99'W	1			2	291
G-71	Along Peach Orchard Rd. under powerline	NC	Scotland	34°55.77'N	079°23.86'W	1			3	292
G-72	Along US 401 and forest edge	NC	Scotland	34°50.49'N	079°23.98'W	1			1	293
G-73	Along forest edge of Hamlet Rd.	NC	Scotland	34°48.01'N	079°38.03'W	1			2	294
G-74	Along Piney Grove Church Rd.	NC	Wayne	35°17.32'N	077°50.92'W	1			1	281
G-75*	Aiken State Park	SC	Aiken	33°32.55'N	081°28.92'W		1		4	324

				Geographic coordinates		Ploidy / Number of plants				
Population	Locality	State	County	Latitude	Longitude	2*x*	4*x*	6*x*	*N*	Voucher no.
G-76*	Parcel at Jct. Hwy 283 & US 1/Columbia Hwy N	SC	Aiken	33°36.11'N	081°41.04'W		1		5	325
G-77	Aiken Gopher Tortoise Heritage Preserve	SC	Aiken	33°30.00'N	081°24.52'W		1		1	231
G-78*	Carolina Sandhills National Wildlife Refuge	SC	Chesterfield	34°31.46'N	080°13.63'W	1			3	331
G-79*	Sandhill State Forest	SC	Chesterfield	34°33.37'N	080°03.84'W	1			3	332
G-80*	H. Cooperblack Jr. Memorial trail/James Rd.	SC	Chesterfield	34°34.03'N	079°55.75'W	1			2	333
G-81	Along Hwy 102	SC	Chesterfield	34°38.30'N	080°05.22'W	1			5	249
G-82	Teals mill Rd./Cheraw State Park	SC	Chesterfield	34°37.25'N	079°56.70'W	1	1		3	250
G-83	W. Old Camden Rd.	SC	Chesterfield	34°22.28'N	080°16.92'W		1		3	252
G-84	US 1	SC	Chesterfield	34°26.17'N	080°17.44'W		1		2	253
G-85	Along Old Stagecoach Rd.	SC	Chesterfield	34°20.96'N	080°21.27'W		1		3	254
G-86	Along Old Georgetown Rd. E.	SC	Chesterfield	34°22.99'N	080°23.29'W		1		1	255
G-87	Co. Rd. S. 18-137	SC	Dorchester	32°54.02'N	080°23.11'W		1		4	248
G-88	Tillman Sand Ridge Heritage Preserve, Sandhill Rd.	SC	Jasper	32°29.69'N	081°11.55'W		1		5	247
G-89*	Along Jefferson Davis Hwy/US 1	SC	Kershaw	34°18.73'N	080°32.49'W					256
G-90*	Goodale State Park	SC	Kershaw	34°17.42'N	080°31.55'W		1		3	329
G-91*	Jefferson Davis Hwy/US 1	SC	Kershaw	34°22.04'N	080°25.92'W		1		4	330
G-92*	Lee State Park	SC	Lee	34°11.81'N	080°11.36'W		1		3	251
G-93	Shealy's Pond Heritage Preserve	SC	Lexington	33°51.82'N	081°14.19'W		1		1	232
G-94	Peachtree Rock Preserve	SC	Lexington	33°49.71'N	081°12.11'W		1		1	233
G-95*	Ft. Jackson, Area 26 B firebreak 16	SC	Richland	34°00.85'N	080°47.40'W		1		2	257
G-96*	Ft. Jackson, Area 34 B near Chauers Pond Rd.	SC	Richland	34°02.36'N	080°43.30'W		1		3	258
G-97*	Ft. Jackson, Area 11 E. of Wildcat Rd.	SC	Richland	34°05.06'N	080°50.61'W		1		2	259
G-98	Ft. Jackson, S. edge of pond of Westons Recreation	SC	Richland	33°59.96'N	080°50.03'W		1		1	260
G-99	Sesquicentennial State Park	SC	Richland	34°05.82'N	080°54.57'W		1		3	326
G-100*	Sesquicentennial State Park	SC	Richland	34°04.92'N	080°54.38'W		1	1	4	327
G-101	Faunas Rd.	SC	Richland	34°08.34'N	081°02.33'W		1		5	328

				Geographic coordinates		Ploidy / Number of plants				
Population	Locality	State	County	Latitude	Longitude	2*x*	4*x*	6*x*	*N*	Voucher no.
G-102*	Forks of River Rd.	VA	Southampton	36°33.85'N	076°55.96'W	1			2	282
G-103	Suffolk City, DCR	VA	Suffolk City	36°33.77'N	076°54.82'W					283
Callisia ornata (Small) G. Tucker										
O-1*	Turkey Creek Sanctuary	FL	Brevard	28°01.01'N	080°36.18'W	1			1	315
O-2*	Sebastian State Park	FL	Brevard	27°50.19'N	080°31.56'W	1			2	361
O-3	Wickham Park	FL	Brevard	28°09.64'N	080°39.54'W	1			1	314
O-4*	Highlands State Park	FL	Highlands	27°28.85'N	081°31.57'W		1		4	301
O-5	Sebring Amtrak Station	FL	Highlands	27°29.75'N	081°26.06'W					298
O-6	Lake June in Winter Scrub State Park	FL	Highlands	27°17.83'N	081°25.14'W		1		2	300
O-7	Little Manatee State Park/Mustang trail	FL	Hillsborough	27°40.08'N	082°22.1'W		1		4	350
O-8	Little Manatee State Park/Dude trail	FL	Hillsborough	27°39.93'N	082°22.38'W		1		3	351
O-9*	Seminole State Forest/entrance Brantley Branch Rd.	FL	Lake	28°53.20'N	081°27.60'W			1	4	343
O-10*	Seminole State Forest/the Simson track	FL	Lake	28°52.94'N	081°31.08'W			1	4	344
O-11*	Seminole State Forest/Warea tract	FL	Lake	28°29.99'N	081°40.03'W			1	3	346
O-12*	Lake Louisa State Park/Primitive campsite	FL	Lake	28°27.17'N	081°44.13'W			1	4	347
O-13	Jonathan Dickinson State Park/Nature trail picnic area	FL	Martin	26°59.58'N	080°08.83'W	1			4	353
O-14*	Tiger Creek Preserve along Pfundstein Rd.	FL	Polk	27°48.41'N	081°29.81'W		1		1	228
O-15*	Arbuckle State Forest, School Bus Rd.	FL	Polk	27°39.75'N	081°23.84'W	1			3	316
O-16*	Lake Kissimmee State Park, Buster Island	FL	Polk	27°55.39'N	081°21.82'W	1			2	354
O-17*	Lake Kissimmee State Park, Catfish Creek	FL	Polk	27°57.84'N	081°22.77'W	1			5	355
O-18*	Lake Kissimmee State Park Main entrance	FL	Polk	27°57.91'N	081°28.34'W	1			5	356
O-19*	Welaka State Forest	FL	Putnam	29°28.24'N	081°39.37'W	1			1	360B
O-20	Dunns Creek State Park entrance Sisco Rd.	FL	Putnam	29°33.34'N	081°34.94'W	1			2	312
O-21	Oscar Scherer State Park along Legacy trail	FL	Sarasota	27°10.17'N	082°27.41'W		1		5	352
O-22*	Tiger Bay State Forest	FL	Volusia	29°10.22'N	081°09.56'W	1			3	313
O-23*	Lake George State Forest	FL	Volusia	29°11.84'N	081°30.55'W			1	1	364

				Geographic coordinates		Ploidy / Number of plants				
Population	Locality	State	County	Latitude	Longitude	2*x*	4*x*	6*x*	*N*	Voucher no.
O-24*	Deland	FL	Volusia	29°00.11'N	081°13.25'W	1			1	365
Callisia rosea (Vent.) D.R. Hunt										
R-1	Along Chert Quarry Rd.	SC	Allendale	33°02.28'N	081°28.26'W	1			3	245
R-2*	Heggie's Rock Preserve	GA	Colombia	33°32.34'N	082°15.09'W	1			3	321
R-3*	Lake Russel State Park	GA	Elbert	34°09.60'N	082°44.42'W	1			3	237
R-4*	Bobbie Brown State Park	GA	Elbert	33°58.35'N	082°34.64'W	1			3	238
R-5*	Elijah Clarke State Park	GA	Lincoln	33°51.22'N	082°24.02'W	1			3	323
R-6	Fort Gordon	GA	Richmond	33°23.49'N	082°14.54'W	1			3	240
R-7	Fort Stewart	GA	Tattnall	32°02.54'N	081°48.84'W	1			4	244

Mature individuals were sampled in the summers of 2012, 2013, 2014, and 2015. Only known localities with collection years between 1970 and 2012 were visited, unless the locality was in a protected area. This approach was used to increase the chances of finding intact populations but meant that we were unable to resample all of Giles's (1942, 1943) locations. Voucher specimens were deposited at the University of Florida Herbarium (FLAS); collection numbers are provided in Table 1.

Population localities were surveyed for individuals with different growth habit and habitat; we then collected across that diversity. Contact zones between species, based on the georeferenced localities, were more intensively surveyed by searching for distinct morphological variation (habit, leaf, and flower) to increase the probability of encountering mixed cytotypes. Two to six live plants were collected per locality. Plants were removed with 15 cm of soil circumference to increase the survival rate and placed in plastic bags. At the Department of Biology, University of Florida greenhouse, plants were then potted in a soil mixture of 1:1 sand and potting soil (Pro-Mix) and were kept under natural light. During the period from December–March, the individuals of putative diploid *C. graminea* and *C. rosea* were given a four-month dormancy treatment at 4°C to mimic their natural habitat.

Chromosome counts

Two individuals per cytotype of *C. graminea* were used as a control for flow cytometry analysis by counting chromosome numbers using established methods (see below). Previous studies of members of Commelinaceae found that cell division in root tips occurs at high frequency during late morning to early afternoon (Faden and Suda 1980). After a series of hourly collections, 2:00 pm was determined to be the optimal time for collecting root tips of *C. graminea*, *C. ornata*, and *C. rosea*.

Root tips were placed in 2 mM 8-hydroxyquinoline following Soltis (1980) for 24 hours at 4°C and then fixed in a 3:1 absolute ethanol-glacial acetic acid solution for 24 hours. Root tips were then placed in 70% ethanol and stored until needed at 4°C. Digestion of the root tips and spreading of the chromosomes on slides were performed following the methods of Kato et al. (2011). Chromosomes were stained with DAPI and visualized using a Zeiss Axio Imager M2 microscope (Carl Zeiss Microscopy LLC, Thornwood, NY, U.S.A.).

Flow cytometry

Preparation of all samples for flow cytometry followed Roberts et al. (2009), in which each sample consisted of approximately 1 cm^2 of fresh leaf tissue of *Callisia*; 0.5 cm^2 dried leaf tissue of *Vicia faba* (26.9 pg) was used as an internal standard (Dolezel et al. 2007). Samples were finely chopped with a sharp single-edged razor blade in a petri dish for 2 min in 1 ml of cold lysis buffer (0.1 M citric acid, 0.5% v/v Triton X–100, 1% w/v PVP–40 in distilled water) (Hanson et al. 2005, Mavrodiev et al. 2015).

After 20–30 sec of incubation on a cold brick that served as a cold chopping surface, each sample was further treated and measured based on the methods of Mavrodiev et al. (2015) on an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, U.S.A). In all, the ploidy of 300 samples was assessed in batches of 28 samples.

For the estimation of genome size, three plants of the same accession were analyzed using the Flow Cytometry Kaluza Analysis Software 1.3 (Beckman Coulter Life Sciences 2016). The relative DNA content was calculated using the ratio of the mean fluorescent peak of the sample to the mean fluorescent peak of the internal standard, multiplied by the genome size of the standard, *Vicia faba* (Dolezel et al. 2007).

Results

Georeferencing and collecting

All GPS points obtained here were incorporated into a map with ARCGIS 10.4 (ESRI 2016) (Figure 1). The results show that *Callisia graminea* ranges from North Carolina to central Florida with an isolated population in southern Virginia. *Callisia rosea* occurs predominantly in South Carolina and Georgia, and *C. ornata* is found in central to southern Florida. Specimens were collected at 133 localities, of which 61 were known from the 436 georeferenced localities and 72 were newly discovered populations. A list of these localities is provided in Table 1, indicating the geographic origin, ploidal level with corresponding number of plants, total number of analyzed individuals, and voucher information for each sample. Illustrations of the habits of diploid *C. graminea*, *C. ornata*, and *C. rosea* are provided in Figure 2.

Chromosome counts

Chromosome numbers were obtained for three individuals per cytotype in *C. graminea*, confirming the presence of $2n = 2x = 12$ (diploids; Figure 3a), $2n = 4x = 24$ (tetraploids; Figure 3b), and $2n = 6x = 36$ (hexaploids; Figure 3c). The diploid and tetraploid counts were obtained for plants from known locations for which previous counts were available (Giles 1942, Kelly 1991). The hexaploids were discovered while counting spreads of putatively tetraploid *C. graminea* from Lake County, FL (Table 1). These $2x$, $4x$, and $6x$ individuals of *C. graminea* were then used as references in subsequent analyses using flow cytometry.

Flow cytometry

Ploidy was estimated via flow cytometry for 300 plants of *C. graminea* (representing 96 populations), *C. ornata* (from 23 populations), and *C. rosea* (from 7 populations). The results and the number of individuals analyzed per population are given in Table

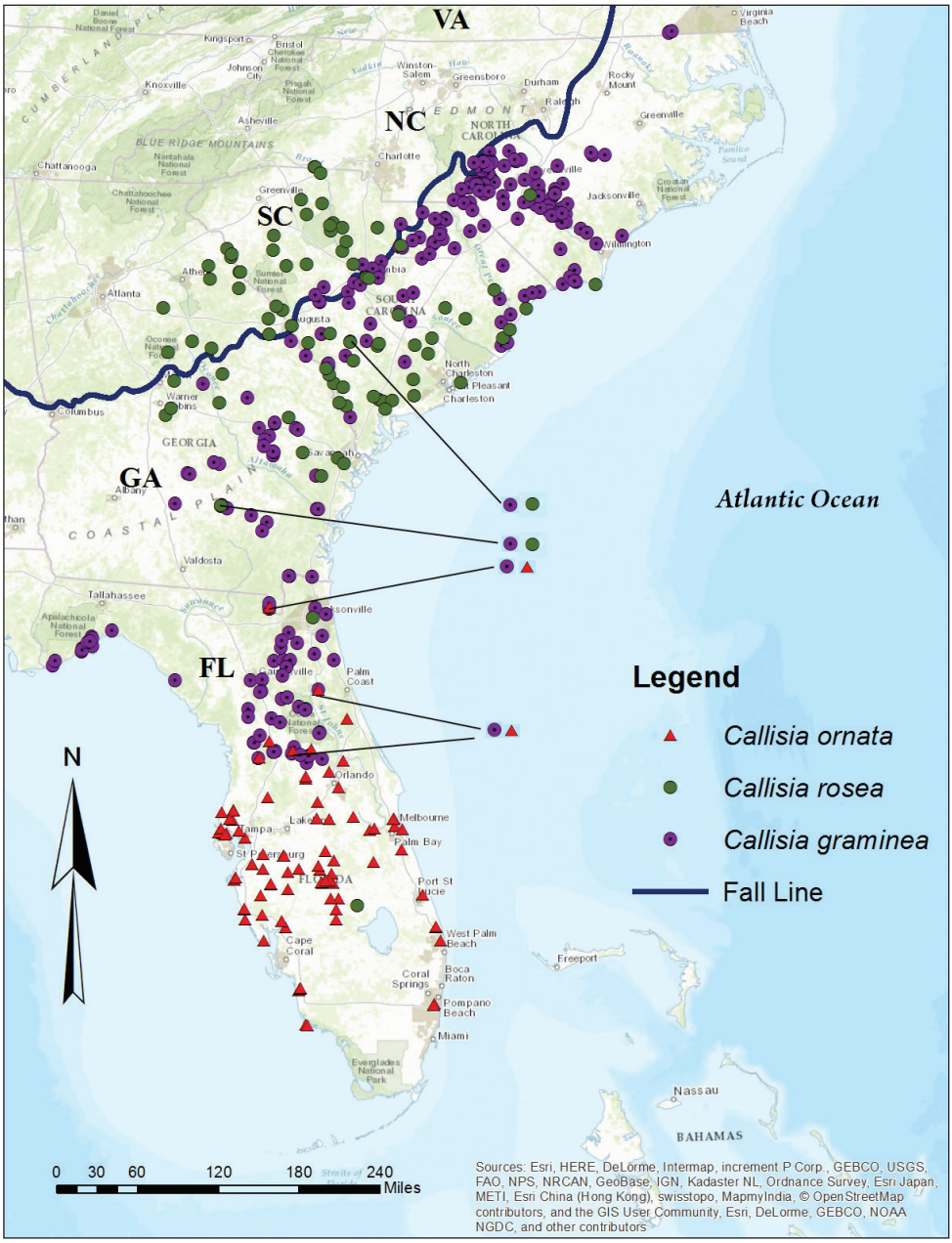

Figure 1. Distribution map of *Callisia* section *Cuthbertia*. Distribution of *Callisia graminea*, *C. ornata*, and *C. rosea* based on georeferenced data. Multiple species occurring in sympatry are designated by superimposed symbols; these locations are further indicated by black lines that highlight the symbols.

1. Three distinct groups of fluorescence intensities were obtained from these analyses that were congruent with chromosome counts of diploid, tetraploid, and hexaploid *C. graminea*. Histograms for the cytotypes of *C. graminea* are shown in Figure 4. Results for 26 individuals (17%) of tetraploid *C. graminea* had a lower fluorescence intensity (sug-

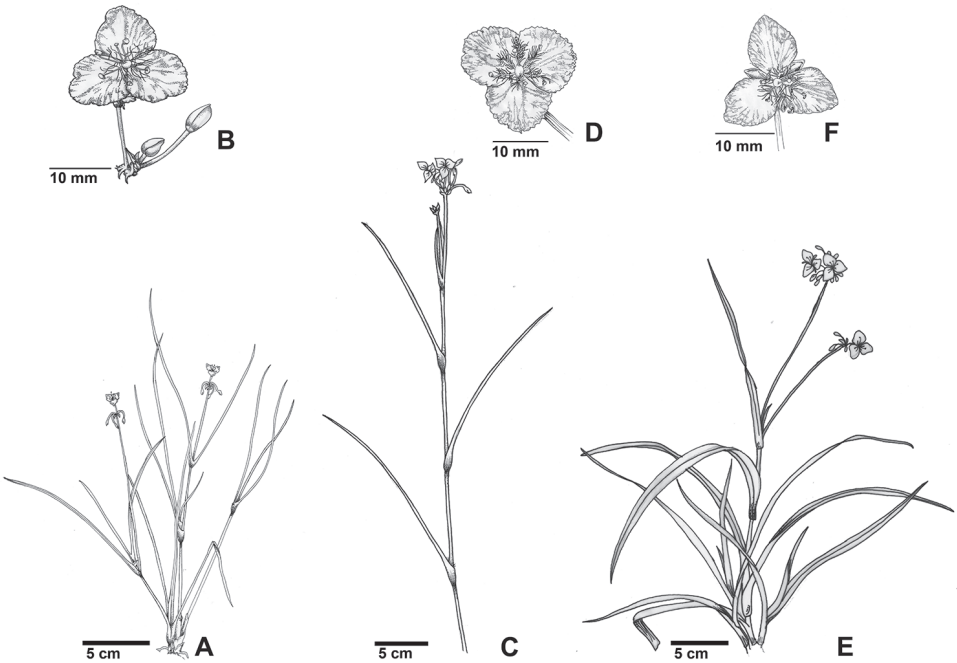

Figure 2. Habit of *Callisia* section *Cuthbertia*. **A** diploid *Callisia graminea* **B** diploid *C. graminea* flower **C** diploid *C. ornata* **D** diploid *C. ornata* flower **E** diploid *C. rosea* and **F** diploid *C. rosea* flower. Illustrations by Sofia Chang.

gesting a smaller genome size) than the remaining 83% of tetraploid *C. graminea*. The ploidy of the former plants was verified by chromosome counts, and all were tetraploid.

The relative genome size of individuals of *C. rosea* was similar to that of diploid *C. graminea* ($2n = 2x = 12$) (see below), confirming that our samples of *C. rosea* are diploid, in agreement with the literature (Giles 1942). Most individuals of *C. ornata* ($2n = 2x = 12$) were also inferred to be diploid, as expected based on previous counts (Giles unpublished), but our analysis also revealed previously unknown tetraploid ($2n = 4x = 24$) and hexaploid populations ($2n = 4x = 36$) of *C. ornata*. The latter were found in Seminole State Forest, FL, where they occur in sympatry with tetraploid individuals of *C. graminea*. All polyploid levels were verified with chromosome counts; chromosome spreads are depicted in Figure 3.

Genome size (2C-value) of cytotypes in *Callisia* section *Cuthbertia* was estimated; data are presented in Table 2 along with previously calculated genome sizes by Hertweck (2011) and Jones and Kenton (1984).

Distribution map – Based on the flow cytometry data, the distribution of cytotypic variation among the 126 populations sampled [*C. graminea* (96 populations), *C. ornata* (23 populations), and *C. rosea* (7 populations)] was mapped (Figure 5). This map shows that diploid *C. graminea* is restricted to two disjunct areas: one in Franklin County, VA, and the second stretching along the Fall Line from North Carolina to South Carolina. Tetraploid *C. graminea* has a broader distribution that runs along the

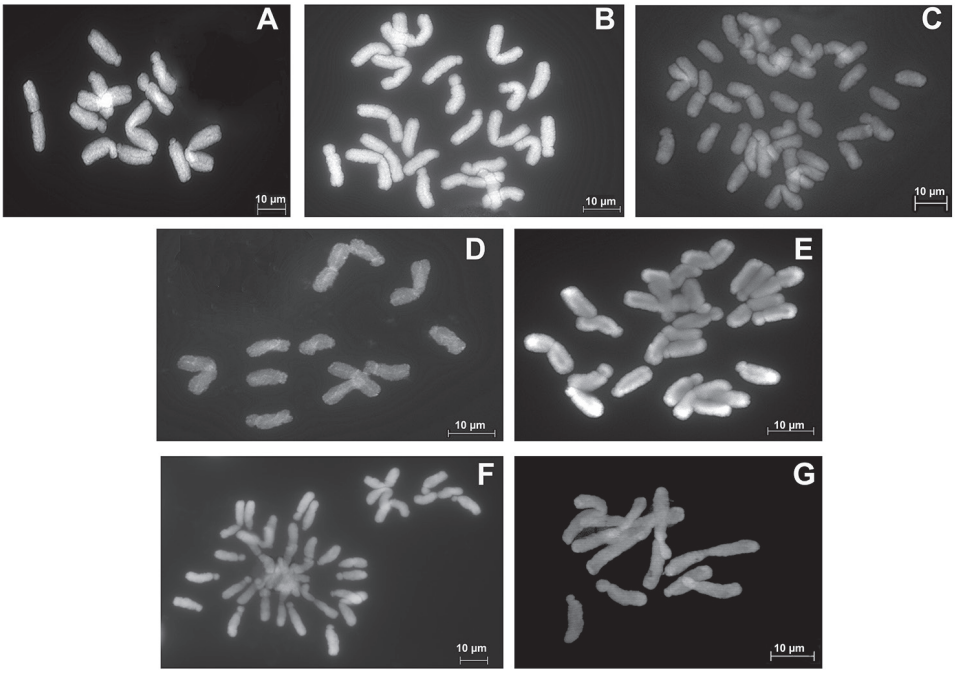

Figure 3. Mitotic metaphase chromosome spreads from root tips. **A** diploid *Callisia graminea* (2*n* = 2*x* = 12) **B** tetraploid *C. graminea* (2*n* = 4*x* = 24) **C** hexaploid *C. graminea* (2*n* = 6*x* = 36) **D** diploid *C. ornata* (2*n* = 2*x* = 12) **E** tetraploid *C. ornata* (2*n* = 4*x* = 24) **F** hexaploid *C. ornata* (2*n* = 6*x* = 36) and **G** diploid *C. rosea* (2*n* = 2*x* = 12).

Table 2. Genome sizes (2C) of *Callisia* section *Cuthbertia* and their cytotypes and previously reported 2C-values. Voucher numbers apply only to the current study.

Species	Chromosomes	2C value (pg)	Hertweck 2011	Jones and Kenton 1984
C. graminea 2*x* (IEM 342)	2*n* = 12	41.75 ± 0.67		
C. graminea 4*x* (IEM 251)	2*n* = 24	78.55 ± 0.42		
C. graminea 6*x* (IEM 236)	2*n* = 36	122.86 ± 0.8		
C. ornata 2*x* (IEM 353)	2*n* = 12	48.51 ± 1.09		
C. ornata 4*x* (IEM 352)	2*n* = 24	87.99 ± 0.4		
C. ornata 6*x* (IEM 349)	2*n* = 36	129.73 ± 0.56		
C. rosea 2*x* (IEM 237)	2*n* = 12	43.70 ± 1.78	43.52	77.3

coastal plain from North Carolina to central Florida. Hexaploid *C. graminea* occurs in Lake and Hernando Counties, FL, and one individual was found in Richland County, SC. In South Carolina, one hexaploid *C. graminea* individual was found growing sympatrically with multiple tetraploid *C. graminea* plants. Based on extensive collecting, our observations suggest that the tetraploid *C. graminea* samples from North Carolina

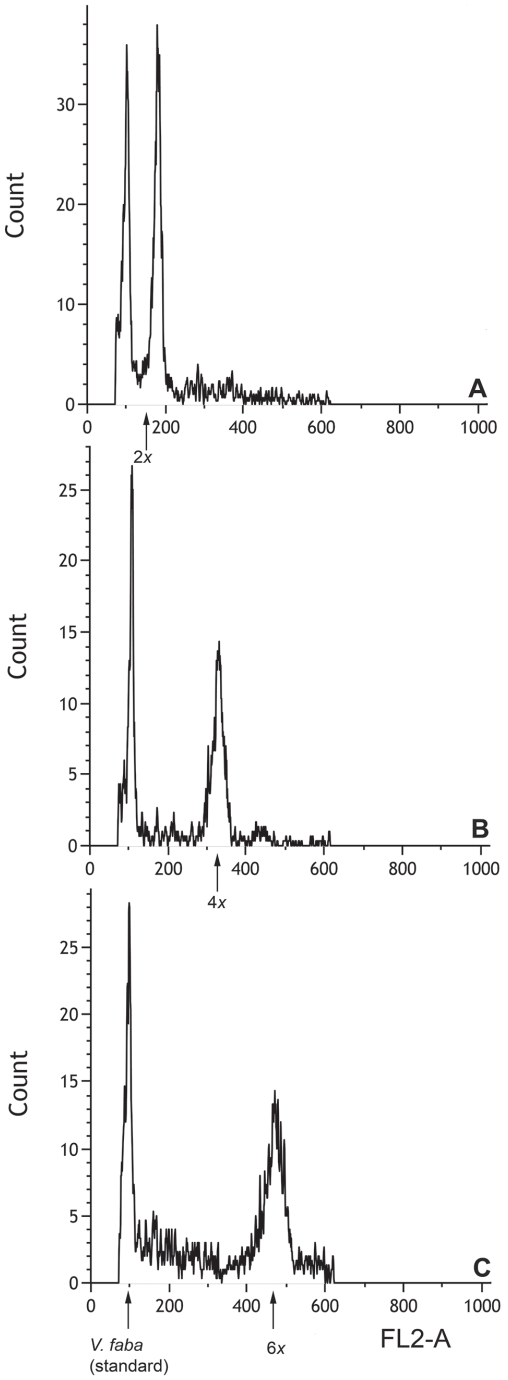

Figure 4. Histograms of fluorescence intensity (FL2-A) of propidium iodide-stained nuclei. **A** diploid *C. graminea* **B** tetraploid *C. graminea* and **C** hexaploid *C. graminea*. *Vicia faba* was used as the internal standard.

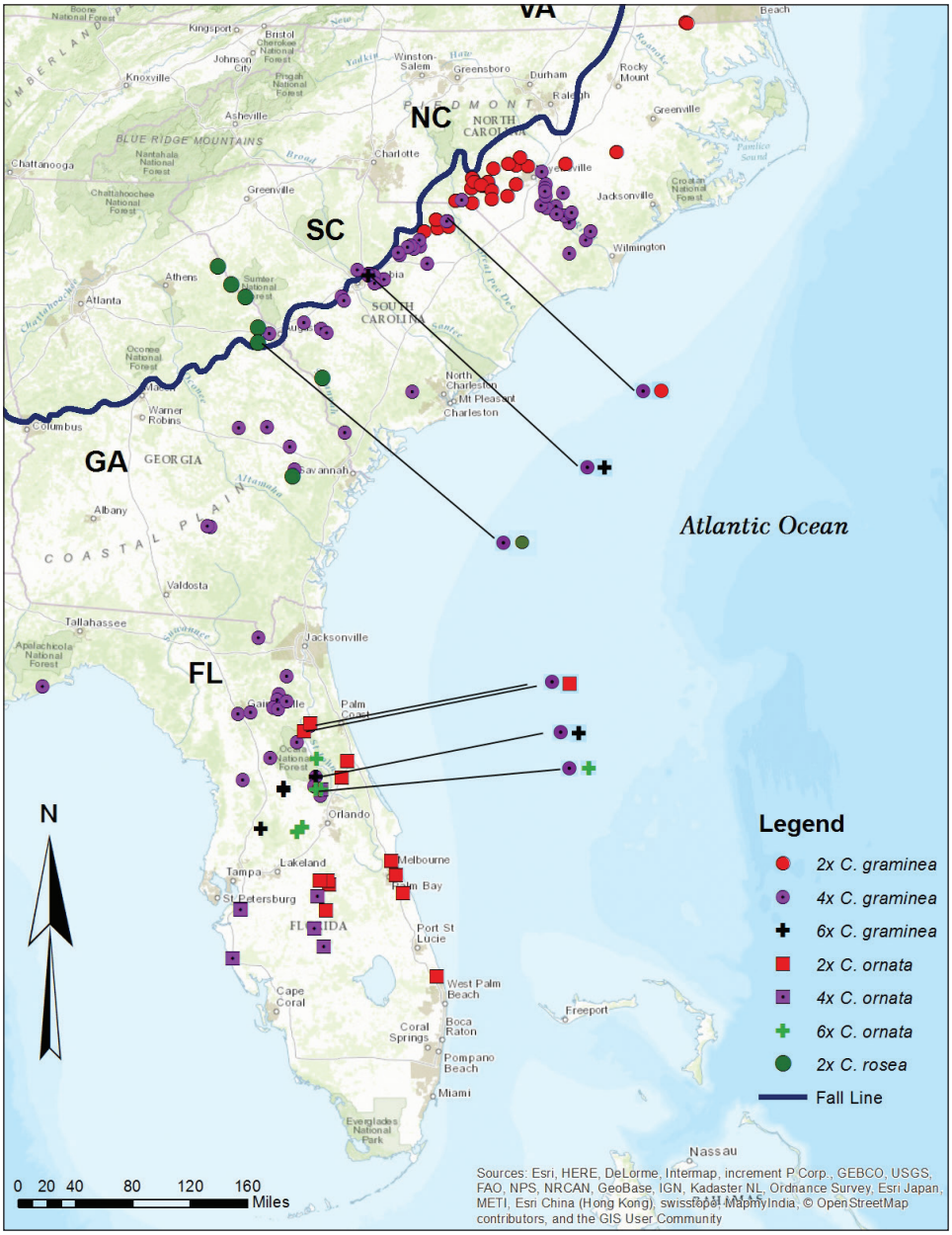

Figure 5. Distribution of cytotypic variation in *C. allisia* section *Cuthbertia*. Diploid *C. graminea* (red circles) ranges from Virginia to North and South Carolina; tetraploid *C. graminea* (purple circles) occurs along the coastal plain from North Carolina to central Florida; hexaploid *C. graminea* (black plus signs) is restricted to central Florida. Diploid *C. ornata* (red squares) occurs in eastern and central Florida; tetraploid *C. ornata* (purple squares) is restricted to central and western peninsular Florida; hexaploid *C. ornata* (green plus signs) is restricted to central Florida. *Callisia rosea* (all diploid; green diamonds) occurs along the Georgia – South Carolina border. Localities with multiple cytotypes or taxa are indicated by black lines. Note: The black plus signs are the hexaploids of *C. graminea,* and the green plus signs are hexaploids of *C. ornata*

are the largest of this species, with clumps that exhibit a diameter of over 25 cm compared to plants in South Carolina, Georgia, and Florida, with a maximum diameter of 15 cm.

Diploid *C. ornata* occurs in eastern Florida (from Putnam through Martin Counties), and tetraploid *C. ornata* occurs in western Florida (Polk, Hillsborough, Highlands, and Lake Counties). Hexaploid *C. ornata* occurs in Lake and Volusia Counties in central Florida.

Diploid *C. rosea* occurs in the piedmont of Georgia and South Carolina with some scattered populations in the coastal plain.

Discussion

Georeferencing – *Callisia* section *Cuthbertia* consists of three species native to the southeastern U.S.A., with three ploidal levels within *C. graminea* and *C. ornata* and diploids in *C. rosea.* The map of the geographic distribution (Figure 1) of all georeferenced voucher specimens depicts all specimens of *C. graminea*, *C. ornata*, and *C. rosea* without ploidal levels, collected from 1894 until present. *Callisia graminea* is the most widely distributed of all species in the genus, ranging from Virginia to Florida. *Callisia ornata* is restricted to Florida; although one specimen was recorded from Charleston County, GA, *C. ornata* was not found in Georgia in this study. *Callisia rosea* occurs mainly in Georgia and the Carolinas, but two herbarium specimens were found from Duval County and Highlands County, FL. The localities of these two herbarium specimens of *C. rosea* were vague, and *C. rosea* was not observed in Florida in this study.

Flow cytometry and genome size – Flow cytometry analysis of ploidal levels in 300 individuals from 126 populations together with 60 additional chromosome counts confirmed the presence of diploid, tetraploid, and hexaploid cytotypes of *C. graminea* and *C. ornata*. Significantly, tetraploid and hexaploid *C. ornata* were previously unknown. Our analysis also confirmed that *C. rosea* is diploid. However, Anderson and Sax (1936) and Ichikawa and Sparrow (1967) reported only tetraploids in *C. rosea*. This might be a misidentification of broad-leaved tetraploid *C. graminea* as *C. rosea*, as suggested by Giles (1942), who only detected diploids in *C. rosea*. Overall, three distinct fluorescent intensity peaks were seen in the histograms among the *C. graminea* and *C. ornata* cytotypes, with peaks for the tetraploids that are approximately twice the size of those of the diploids and for the hexaploids that are approximately three times those of the diploids. This general pattern of genome size increase in polyploids is to be expected relative to their diploid progenitors (Leitch and Bennett 2004).

It is interesting to note that 26 individuals (17%) of tetraploid *C. graminea* had a lower fluorescence intensity than the remaining 83%, suggesting a smaller genome size. The individuals with the smaller peak than that typical of other tetraploids were measured twice with the flow cytometer, and the results were consistent. The chromosome numbers

of these samples were verified by chromosome counts, and all were tetraploid ($2n = 4x = 24$). Reductions in genome size in polyploids are common (Leitch and Bennett 2004), and in this study two hypotheses are possible: genome downsizing or the occurrence of multiple origins from parents having different genome sizes. Because this variation in genome size occurs among individuals within populations and because the individuals are not clustered in a single geographic area, we suggest that this variation in DNA content might be a result of genome downsizing, but this hypothesis requires further testing.

Genome size can be used, with other methods, to hypothesize putative progenitors of polyploids (e.g. Eilam et al. 2010). In diploid *C. graminea* the estimated 2C-value is 41.75 pg; the value for tetraploid *C. graminea* is 78.55 pg. According to Giles (1942), multivalent chromosome pairing was observed in tetraploid *C. graminea,* suggesting autopolyploidy. If tetraploid *C. graminea* is of autopolyploid origin, the expected DNA content would be 83.47 pg, but the observed DNA content of tetraploid *C. graminea* is 4.95 pg lower than the expected 2C-value. Newly formed polyploids usually possess a DNA content equal to the sum of the 2C-values of their progenitors (Bennett et al. 2000, Eilam et al. 2010). Over time, however, genome downsizing in polyploids relative to their progenitors is expected (Leitch and Bennett 2004), which seems to be the case in tetraploid relative to diploid *C. graminea*.

Due to the rarity of hexaploid *C. graminea* in South Carolina, we only calculated the 2C-value of hexaploids that occur in Florida. Hexaploid *C. graminea* may be of allo- or autopolyploid origin. If from allopolyploid origin, the expected 2C-value would be 127.06 pg, with diploid *C. ornata* (48.51 pg) and tetraploid *C. graminea* (78.55 pg) as the progenitors. The observed genome size of hexaploid *C. graminea* is 122.86 pg, which is lower than the expected value, again consistent with genome downsizing. In the case of an autopolyploid origin with tetraploid *C. graminea* (78.55 pg) as parent, we would expect a genome size of 117.83 pg, which is approximately 5 pg less than the observed 2C-value. Genome size data do not conclusively elucidate the origins of hexaploid *C. graminea*; both allo- and autopolyploidy are possible, and its origin requires further testing. However, Giles (1942) noted multivalent formation, generally indicative of autpolyploidy, in hexaploid *C. graminea*.

Tetraploid *C. ornata* has a 2C-value of 87.99 pg. It could be of autopolyploid origin with diploid *C. ornata* (48.51 pg) as the parent given that no other extant taxa are sympatric with it. However, the expected DNA content (97.02 pg) is at least 9 pg higher than observed; in contrast, when considering tetraploid *C. ornata* as a possible allopolyploid with tetraploid *C. graminea* (78.55 pg) and diploid *C. ornata* (48.51 pg) as parents (based on an unreduced gamete of the latter), the results (87.79 pg) are similar to the observed DNA content. These results therefore support allopolyploidy over autopolyploidy, yet further analyses are needed to clarify the origin of this cytotype.

Hexaploid *C. ornata* could be of allo- or autopolyploid origin. If allopolyploid, the expected genome size would be 127.06 pg with diploid *C. ornata* (48.51 pg) and tetraploid *C. graminea* (78.55 pg) as parents. The observed DNA content is 129.73 pg, which is slightly higher than the expected 2C-value. Alternatively, it could be an allohexaploid between tetraploid *C. ornata* (87.99 pg) and diploid *C. graminea* (41.75 pg), with an expected genome size of 129.74 pg, essentially identical to the observed

value. In the case of autopolyploidy, we calculated an expected 2C-value of 145.53 if the value is 3 times that of diploid *C. ornata* (48.51 pg), 136.5 pg if tetraploid (87.99 pg) and diploid (48.51 pg) *C. ornata* are considered the parents, and 131.99 pg if a reduced and unreduced gamete of tetraploid *C. ornata* yield the hexaploid. The latter case is closest to the observed value, suggesting either that hexaploid *C. ornata* is of allopolyploid origin, or if an autopolyploid, it arose via the third possible mechanism outlined above; these hypotheses require further investigation.

Based on the Plant DNA C-values Database, http://data.kew.org/cvalues/ (Bennett and Leitch 2012), recorded species of Commelinaceae have a minimum 2C-value of 5.16 pg for *Commelina erecta* L.1753 and a maximum of 86.7 pg for *Tradescantia virginiana* L. 1753. The DNA content of hexaploid *C. graminea* and hexaploid *C. ornata* are currently the highest within Commelinaceae and Commelinales (Leitch et al. 2010) with 122.86 pg and 129.73 pg, respectively. Jones and Kenton (1984) reported that the 2C-value of *C. rosea* is 77.3 pg, with a chromosome count of $2n = 24$, consistent with tetraploidy reported by Anderson and Sax (1936) and Ichikawa and Sparrow (1967); however, as noted above, Giles (1942) only detected diploids ($2n = 12$) for *C. rosea*, consistent with our results. The closest 2C-value to 77.3 pg is the 2C-value of tetraploid *C. graminea* with 78. 55 pg and $2n = 24$ chromosomes; tetraploid *C. graminea* plants with broad leaves may be misidentified as *C. rosea* (Giles 1942). A voucher specimen of *C. rosea* from Jones and Kenton (1984) was not reported, so we cannot assess if the plant material used for the DNA content analysis was identified correctly. A misidentification is likely since the genome size estimation of Hertweck (2011) is close to our values. Likewise, previous tetraploid counts (Anderson and Sax 1936, Ichikawa and Sparrow 1967, Jones and Kenton 1984) may also be for tetraploid *C. graminea* plants that were misidentified as *C. rosea*. Alternatively, there may be cryptic tetraploidy in *C. rosea* that we failed to detect, but given our extensive sampling, we do not believe this to be the case.

Distribution – As shown in Figure 5, two isolated populations of diploid *C. graminea* were detected. One population is in Suffolk County, VA, and the other is in North and South Carolina. These two isolated populations may have been part of a once larger geographic range for diploid *C. graminea,* but due to heavy agricultural activities in this part of North Carolina, suitable habitats ranging from Johnston County to Northampton County were transformed to farmland (personal observation). This anthropogenic influence may have caused the separation of the two isolated groups of diploid *C. graminea*.

Tetraploid *C. graminea* ranges from the coastal plain of the Carolinas to central Florida, with additional populations in the Florida panhandle (Franklin County, FL). This cytotype is clearly more abundant than diploid *C. graminea*; it is usually found in xeric disturbed areas and exhibits a larger growth form than diploid *C. graminea*. These tetraploids were abundant in Bladen and southern Cumberland Counties, NC, which border the isolated locality of diploid *C. graminea* in North Carolina. These two areas (occupied by tetraploid and diploid plants, respectively) are separated by the city of Fayetteville, NC. Although diploid and tetraploid entities of *C. graminea* were reported to be geographically isolated (Bergamo 2003, Giles 1942, 1943, Kelly 1991), one tetraploid individual was found within a diploid population in Cheraw State Park,

SC; this individual is morphologically similar to the surrounding diploid *C. graminea*. This finding supports Giles's (1942) hypothesis that tetraploid *C. graminea* is an autotetraploid because it occurs consistently with diploid *C. graminea*. This hypothesis requires testing with molecular data.

The Fall Line runs essentially east-west through Georgia and from southwest to northeast in the Carolinas. Diploid *C. rosea* occurs on both sides of the Fall Line from Georgia to North Carolina. In Fort Gordon (Richmond County, GA), diploid *C. rosea* occurs in sympatry with tetraploid *C. graminea*. Although these two species occur in sympatry, hybrids were not observed at the site.

Diploid *C. ornata* is endemic to Florida, and tetraploid individuals of *C. ornata* occur in western Florida. These individuals may be autopolyploid, with diploid *C. ornata* as their progenitor. The distribution map in Figure 5 clearly supports the assumption of autopolyploidy, because there are no other *Callisia* species recorded in the region of diploid and tetraploid *C. ornata*. Morphologically, tetraploid *C. ornata* individuals show an increased axillary branching pattern, which is less common in diploid individuals. Axillary branching is a characteristic of *C. graminea.* Tetraploid *C. graminea* and diploid *C. ornata* are likely parents, through the union of one reduced gamete of tetraploid *C. graminea* and one unreduced gamete of diploid *C. ornata.*

In South Carolina, one hexaploid individual of *C. graminea* was found growing sympatrically with multiple tetraploid individuals of *C. graminea*. Hexaploid *C. graminea* in South Carolina appeared to be rare, and in 1942 only one individual was reported by Giles (1942). These rare hexaploid individuals may be allopolyploids, with diploid *C. rosea* and tetraploid *C. graminea* as their parents or autopolyploids with tetraploid *C. graminea* as their progenitor. Regarding allopolyploidy, *C. rosea* was not found sympatrically with tetraploid *C. graminea* in South Carolina; however, from the map of georeferenced specimens (Figure 1), there is a significant overlap of distribution between tetraploid *C. graminea* and diploid *C. rosea* in the Carolinas. With regard to autopolyploidy, individuals may have resulted through the union of one reduced and one unreduced gamete of tetraploid *C. graminea* given that no other *Callisia* species were observed in the population.

In Lake and Hernando Counties, FL, hexaploid individuals exhibited intermediate morphological characteristics between *C. graminea* and *C. ornata*. Some populations had typical tetraploid *C. graminea* or diploid *C. ornata* characteristics (Figure 2). Two forms were distinguished based on habit: (1) hexaploid *C. graminea* and (2) hexaploid *C. ornata*. Hexaploid *C. graminea* and one of its possible progenitors, tetraploid *C. graminea*, grow in sympatry at the Seminole State Forest, and hexaploid *C. ornata* was found growing with tetraploid *C. graminea* at the entrance to Brantley Branch Rd. (Seminole State Forest). The co-occurrence of hexaploids and tetraploids suggests that the hexaploids may be of allopolyploid origin. Hexaploid *C. graminea* was also collected at Lake Griffin State Park, Edward Rd., Lady Lake, and Seminole State Forest, FL. In Dunns Creek State Park and Welaka State Forest, diploid *C. ornata* and

tetraploid *C. graminea* occur in sympatry; however, hexaploids were not found in these contact zones.

The rare hexaploid collected in South Carolina is most likely independently evolved from the hexaploids from Florida, and this entity from South Carolina could be either an allo- or autopolyploid. If allopolyploid, one likely parent, *C. rosea,* only occurs in Georgia and the Carolinas; if autopolyploid, the likely parent is tetraploid *C. graminea.* The hexaploid entities of Florida might be allopolyploid due to the intermediate morphological characters, with diploid *C. ornata* and tetraploid *C. graminea* as progenitors.

Callisia graminea forma *leucantha*, which was reported near Tampa, FL, was not found, but one white-flowered tetraploid individual of *C. graminea* was encountered among pink-flowered individuals in each of the following three locations: Sesquicentennial State Park, SC; Chesterfield Co., SC; and Tate's Hell State Forest, FL. One white-flowered individual of diploid *C. rosea* was found in Heggie's Rock Preserve, Appling, GA. White flowers reflect an absence of anthocyanins, which may result from mutations in any of the genes in the anthocyanin pathway or from lack of expression of potentially functional genes (Ho and Smith 2016, Rausher 2008). In *Callisia* section *Cuthbertia*, variation in flower color is common, but there is no association between color and ploidy within or among populations. Loss of anthocyanin pigments seems to occur sporadically within this complex.

Morphological and molecular analysis is an important next step in unraveling the complex relationships among cytotypes of *Callisia* section *Cuthbertia.* This work will allow us to reveal the parentage, evolutionary history, and the evolutionary role of all cytotypes within *Callisia* section *Cuthbertia*.

Acknowledgments

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Molecular cytogenetic characterization and comparison of the two cultivated *Canavalia* species (Fabaceae)

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Abstract

The two cultivated *Canavalia* (Adanson, 1763) species, *Canavalia gladiata* (N. J. von Jacquin, 1788) A. P. de Candolle, 1825 and *Canavalia ensiformis* (Linnaeus, 1753) A. P. de Candolle, 1825 are closely related based on morphological and molecular phylogenetic data. However, the similarities and differences in genome organization between them have not been evaluated at molecular cytogenetic level. Here, detailed karyotypes of both species were constructed using combined PI and DAPI (CPD) staining, rDNA-FISH and self-genomic *in situ* hybridization (sGISH). For further comparison, comparative genomic *in situ* hybridization (cGISH) and sequence analysis of 5S rDNA were applied. Their chromosomes were accurately identified by sGISH and rDNA-FISH signals. Both species had the karyotype formula 2n = 22 = 18m + 4m-SAT, but the karyotype of *C. ensiformis* was shorter and more asymmetric than that of *C. gladiata.* They displayed similar CPD bands at all 45S rDNA sites and centromeres. *C. gladiata* had ten centromeric 5S rDNA loci and two SC (secondary constriction)-associated 45S rDNA loci. *C. ensiformis* had nine centromeric and one interstitial 5S loci, two SC-associated and one proximal 45S loci. Their sGISH signal patterns displayed both basic similarities and distinct differences. Reciprocal cGISH generated prominent signals in all pericentromeric regions and 45S sites. There was lower level of sequence identity of the non-transcribed spacer between their 5S rDNA repeats. These data confirmed the evolutionary closeness between *C. gladiata* and *C. ensiformis* and demonstrated obvious differentiation between their genomes, and supported the opinion that *C. ensiformis* is more advanced in evolution than *C. gladiata*.

Keywords

Canavalia cultivars, karyotype, 5S rDNA, 45S rDNA, fluorochrome banding, *in situ* hybridization

Introduction

The genus *Canavalia* Adanson, 1763, belonging to the tribe Diocleae of the family Fabaceae, comprises about sixty pantropical species (Smartt 1990, Snak et al. 2016). This genus has two cultivated species, *Canavalia gladiata* (N. J. von Jacquin, 1788) A. P. de Candolle, 1825 (sword bean) and *Canavalia ensiformis* (Linnaeus, 1753) A. P. de Candolle, 1825 (jack bean). *C. gladiata* was domesticated in Asia and widely cultivated in the tropics whereas *C. ensiformis* is native to Central America and the West Indies and is widely cultivated in tropical and subtropical regions (Smartt 1990). Both are raised as food, forage, green manure, and cover crops to control erosion (Smartt 1990, Ekanayake et al. 2000). Their young seeds and immature pods are cooked and eaten as vegetables. The seeds of *C. gladiata* are used in Chinese herbal medicine as a treatment for cold, hiccups and vomiting (Chinese Pharmacopoeia Commission 2015). The seeds of *C. ensiformis* are a source of concanavalin A (Morris 2007).

Although *C. gladiata* and *C. ensiformis* differ in geographical origin, they are closely related. This fact was established by their highly similar morphologies and seed proteins (Smartt 1990), and the molecular phylogenetic tree (Snak et al. 2016). Purseglove (1974) suggested that *Canavalia virosa* (Roxburgh, 1814) Wight & Arnott, 1833, a wild bean found in tropical Asia and Africa, is the ancestral form of *C. gladiata*. No such progenitor has been suggested for *C. ensiformis* among New World species. Westphal (1974) suggested that *C. gladiata*, *C. ensiformis*, and *C. virosa* are so morphologically similar that in effect they constitute a single species. Therefore, they may, in fact, be geographical or domesticated races within a single biological species (Smartt 1990). Testing these hypotheses at cytogenetic and molecular levels is straightforward. However, there is very little cytogenetic and molecular data available for *Canavalia* spp. To date, cytogenetic studies on *C. gladiata* and *C. ensiformis* have been limited to karyomorphological descriptions of conventionally stained metaphase chromosome complements (Bhandari et al. 1969, Bairiganjan and Patnaik 1989, Li 1989, Rodrigues and Torne 1990, Chen 2003). The genome organization of the two species has not yet been determined using fluorochrome banding and fluorescence *in situ* hybridization (FISH).

Detailed karyotypes displaying chromosome morphology, heterochromatin distribution, and location of repetitive DNA sequences and bacterial artificial chromosome (BAC) have been constructed for many plant species. These are used to reveal chromosome-level genome organization, investigate the evolutionary relationships among related species, and integrate genetic and physical maps (Fuchs et al. 1998, Moscone et al. 1999, Hasterok et al. 2001, de Moraes et al. 2007, Hamon et al. 2009, Robledo et al. 2009, Fonsêca et al. 2010, Chacón et al. 2012, She et al. 2015, She and Jiang 2015, Zhang et al. 2015, Kirov et al. 2016). Karyotype analysis is often hampered by limitations in the ability to identify individual chromosomes due to a lack of markers. To overcome this obstacle, chromosome banding techniques such as Giemsa banding, fluorochrome banding, and FISH using repetitive DNA sequences and BAC clones as probes have been successively applied.

Combined propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) staining (CPD staining; a type of fluorochrome banding) simultaneously reveals GC- and AT-rich chromosome regions (Peterson et al. 1999, Chaowen et al. 2004, She et al. 2006, She et al. 2015). The rRNA genes, 5S and 45S (18S-5.8S-26S) rDNA, have been widely applied in plants as repetitive DNA probes for FISH. The 45S rDNA is present in hundreds of repeated units arranged in tandem arrays. The 5S rDNA is also arranged in tandem arrays of hundreds to thousands of copies. Each 5S rDNA repeat unit consists of a coding region and a non-transcribed spacer (NTS). The coding region is approximately 120 bp and highly conserved across species. In contrast, the NTS regions show much intra- and inter-specific variability in length or nucleotide composition (Sastri et al. 1992). The NTS sequences of 5S rDNA have been used to study phylogenetic relationships among infrageneric taxa (Liu et al. 2003). The distribution patterns of rRNA genes revealed by FISH can be used as karyotype markers (Moscone et al. 1999, Hasterok et al. 2001, Chacón et al. 2012, She et al. 2015, She and Jiang 2015, Kirov et al. 2016). In a phylogenetic context, interpreting the changes in the number and location of rDNA loci in related species facilitates the understanding of the mechanisms and directions of chromosomal changes and their impact on plant evolution (e.g. Moscone et al. 2007, de Moraes et al. 2007, Chung et al. 2008, Weiss-Schneeweiss et al. 2008, Hamon et al. 2009, Robledo et al. 2009, Wolny and Hasterok 2009, She et al. 2015).

The GISH technique, a modification of FISH using genomic DNA as a probe, is conventionally utilized for identifying parental genomes in hybrids and allopolyploids (Schwarzacher et al. 1989). Two adaptations of the GISH technique, self-genomic *in situ* hybridization (sGISH) and comparative genomic *in situ* hybridization (cGISH), have been developed for plant genome analysis. In sGISH, the genomic DNA of a species is applied to its own chromosomes. It is an effective way to reveal the chromosomal distribution of repetitive DNA sequences in a given species (She et al. 2007, Falistocco and Marconi 2013, Zhang et al. 2015). In some plants, sGISH signal patterns permitted accurate identification of entire chromosomes or portions of them (She et al. 2007, Zhou et al. 2008, Zhang et al. 2015). In cGISH, the labeled total genomic DNA of one species is hybridized to the chromosomes of another species without the competitive DNA. It generates hybridization signals in the chromosomal regions of conserved repetitive DNA sequences. Therefore, it can be used to identify the phylogenetic relationships among related species (Falistocco et al. 2002, Wolny and Hasterok 2009, She et al. 2015, Zhang et al. 2015).

In the present study, molecular cytogenetic characterization of *C. gladiata* and *C. ensiformis* was performed using sequential CPD staining, dual color FISH with 5S and 45S rDNA probes, and sGISH. Detailed karyotypes of the two species were established using a combination of chromosome measurements, CPD bands, and rDNA-FISH and sGISH signals. cGISH of the genomic DNA of one species to the chromosomes of the other species was also performed. The 5S rDNA repeats of the two species were cloned, sequenced, and mapped using FISH. The data were assessed to gain insights into the evolutionary relationships between the two cultivated *Canavalia* species.

Material and methods

Plant materials and genomic DNA extraction

Seeds of *C. gladiata* (Jacq.) DC. were obtained from the Chinese Crop Germplasm Resources Information System (CGRIS) and collected in China. Seeds of *C. ensiformis* (L.) DC. were kindly provided by the United States (US) National Plant Germplasm System (NPGS) and collected in Brazil (PI 337078). For GISH and amplification of the 5S rDNA sequences, genomic DNA (gDNA) was extracted from young leaves using cetyltrimethylammonium bromide (CTAB) based on the method described by Murray and Thompson (1980).

Amplification, cloning, and sequencing of 5S rDNA

The 5S rDNA sequences (including the coding regions and NTS) were amplified by polymerase chain reaction (PCR) using the specific primers 5S1 (5' -GGATGGGT-GACCTCCCGGGAAGTCC-3') and 5S2 (5' -CGCTTAACTGCGGAGTTCT-GATGGG-3') deduced from the 5S rRNA gene coding sequence of *Beta vulgaris* Linnaeus, 1753 (Schmidt et al. 1994). The PCR profile was as follows: denaturation at 94°C (3 min); 35 cycles at 94°C (1 min), 56°C (45 s), and 72°C (90 s); extension at 72°C for 10 min. The gel was purified using a PCR Product Purification Kit (Sangon Biotech, Shanghai, China). The PCR products were then ligated to pUCm-T vector using a Sangon Biotech PCR Cloning kit, transformed into *Escherichia coli* JM109 competent cells, and plated on selective medium with ampicillin. Clones were directly screened by PCR for the presence of inserts of the expected size. Five clones per species were amplified using the M13 forward and reverse primers then sequenced using the ABI PRISM 3730 DNA sequencer (Sangon Biotech). The DNA sequences of the five clones from each species were aligned to generate consensus sequences. Similarity searches were conducted on the BLAST site (http://blast.ncbi.nlm.nih.go) of the NCBI database. Using the ClustalW program in MEGA 4.0 (Tamura et al. 2007), the DNA sequences were aligned and the G + C content and variable sites were analyzed.

Chromosome preparations

The procedure for mitotic chromosome preparation was essentially the same as that reported in published protocols (She et al. 2015). Seeds were germinated in the dark at 28°C on filter paper moistened with tap water. Actively growing root tips were pretreated with saturated α-bromonaphthalene for 1.0 h at 28°C then fixed in 3:1 (v/v) methanol/glacial acetic acid overnight. The root tips were then washed in double-distilled water and citrate buffer (0.01 mM citric acid-sodium citrate, pH 4.6) for 10 min each and incubated in a mixture of 1% cellulase RS (Yakult Pharmaceutical Indus-

try, Tokyo, Japan), 1% pectolyase Y23 (Yakult Pharmaceuticals), and 1% cytohelicase (Sigma-Aldrich, Steinhem, Germany) in citric acid buffer at 28°C for 1.5 h. Root tips were transferred to a glass slide along with the fixative and dissected using fine-pointed forceps. Finally, the slides were dried above a flame and stored at −20°C.

Staining with CPD

The CPD staining followed the procedure described in She et al. (2006). Chromosome preparations were treated with RNase A and pepsin then stained with a mixture of 0.6 μg ml^{-1} PI and 3 μg ml^{-1} DAPI (both from Sigma-Aldrich) in a 30% (v/v, using double-distilled water as solvent) solution of Vectashield H-1000 (Vector Laboratories Burlingame, USA). Preparations were examined under an Olympus BX60 epifluorescence microscope equipped with a CoolSNAP EZ CCD camera (Photometrics, Tucson, USA). The CCD camera was controlled using MetaMorph software (Molecular Devices, Sunnyvale, USA). Observations were made and photographs taken using a green excitation filter for PI and a UV excitation filter for DAPI. Greyscale images of each same plate were merged to produce a CPD image. The final images were optimized for contrast and background using PHOTOSHOP version 8.01 (Adobe).

Probe DNA labeling

A 45S rDNA clone containing a 9.04-kb tomato 45S rDNA insert (Perry and Palukaitis 1990) and a pTa794 clone containing a 410-bp BamHI fragment of wheat 5S rDNA (Gerlach and Bedbrook 1979) were used as probes to localize the two ribosomal RNA gene families. They were labeled with biotin-16-dUTP and digoxigenin-11-dUTP, respectively, using Nick Translation Kit (Roche Diagnostics, Mannheim, Germany). The cloned 5S rDNA repeats and the gDNA from *C. gladiata* and *C. ensiformis* were labeled with digoxigenin-11-dUTP using the Nick Translation Kit. Approximately 1 μg plasmid or genomic DNA was used to label each probe.

Fluorescence *in situ* hybridization

FISH with 5S and 45S rDNA probes and cGISH were carried out after CPD staining on the same slides. FISH with cloned 5S rDNA repeats and sGISH were conducted on the slides that were previously stained with CPD and hybridized with the 5S and 45S rDNA probes. The slides were then washed in 2× SSC (Saline-sodium citrate buffer) twice for 15 min each, dehydrated through an ethanol series (70%, 90%, and 100%, 5 min each), and used for hybridization. The *in situ* hybridization procedure followed the protocol described in detail by She et al. (2006). The biotin-labeled probe was detected using Fluorescein Avidin D (Vector Laboratories). The digoxigenin-labeled

probe was detected by Anti-digoxigenin-rhodamine (Roche Diagnostics). Slides were counterstained and mounted with 3 μg ml^{-1} DAPI in 30% (v/v) Vectashield H-1000 and examined under an epifluorescence microscope fitted with a CCD camera. The chromosome spreads recorded in previous CPD and FISH experiments were examined. Grey-scale images were digitally captured using MetaMorph software with UV, blue and green excitation filters for DAPI, fluorescein, and rhodamine, respectively. The images were then merged and edited with PHOTOSHOP version 8.01 (Adobe).

Karyotype analysis

For each species, five metaphase plates that had been subjected to sequential CPD staining, rDNA-FISH, and sGISH were measured using Adobe Photoshop version 8.01 to obtain chromosome relative lengths (RL; percentage of haploid complement), arm ratios (AR; long arm/short arm), fluorochrome band and sGISH signal sizes, and percent distance from the centromere to the rDNA site (di = $d \times 100/a$; where d = distance from the middle of the rDNA sites to the centromere; a = corresponding chromosome arm length). The satellite length was included in the respective chromosome arm length. The stretched secondary constriction (SC) lengths were omitted. The total haploid complement length (TCL; the karyotype length) was measured using the five metaphase cells with the highest degree of chromosome condensation. The arm ratios were used to classify the chromosomes according to the system described by Levan et al. (1964). Chromosomes were identified and idiograms were drawn based on the measurements, fluorochrome bands, rDNA-FISH signals, and sGISH signals. The chromosomes in the karyotype were arranged by order of decreasing size. Karyotype asymmetry was determined using the mean centromeric index (CI), the intrachromosomal asymmetry index (A1), the interchromosomal asymmetry index (A2) (Romero Zarco 1986), the ratio of long arm length in chromosome set to total chromosome length in set (As K%) (Arano 1963), the asymmetry index (AI) (Paszko 2006), and the categories of Stebbins (1971).

Results

Characterization of 5S rDNA repeats

For both species, genomic DNA amplification produced one major fragment of approximately 950 bp and one minor fragment of approximately 450 bp. Amplicons were cloned. Ten from each transformation were screened to verify the presence of the insert. Five clones of each fragment were sequenced.

Sequence analysis showed that all inserts correspond to 5S rDNA repeats. Each fragment was neighbored by 40 bp and 58 bp of the gene at the 5' and the 3' ends, respectively (Fig. 1). There was complete homology among the transcribed regions of

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C. e. GGATGGGTGA CCTCCCGGGA AGTCCTCGTG TTGCAGCTCC TCCGTTTTGG  [ 50]
C. g. .......... .......... .......... .......... ...T.....C  [ 50]

C. e. GTCGGTCGGG CTTATTCTCT TGGTGGCGCG CACTCGCATG GTTTTGATTT  [100]
C. g. ...A...... A..G....T. A......... ...G...... ....C....G  [100]

C. e. CCGAAATCCA TCACGGCGGC GGAGGCAGGC CCGGGACCGC GGCAGTTCGG  [150]
C. g. .TC......T ..T....... ......G... .......... .....G....  [150]

C. e. TTTCCTCACC CGCCGGCTCC GATCCTTGGC GTTGCCGCAA AAGCTGCGAA  [200]
C. g. .C.......T ..GT...... .......... .....T.... .....C....  [200]

C. e. ACGGGCCCCG GAATGGGTTT CTGTGGTCGA GGATATCCTC AACCGGGCGA  [250]
C. g. .......... ...CA..... ...G...... ..TCGA.... .........G  [250]

C. e. GCCCAGGTTG GGACGGCGGC GGGCTCAGGA CGAGGTTTCC AGCGGCCCCT  [300]
C. g. ....C..... .......... .......... ....C..... ......G.T.  [300]

C. e. CCCGAGCCTC GAGATTCATC CCGGACAGAA AGTTATGATC ATTTGAAGCG  [350]
C. g. ....G....T .......... .......... .......... G........  [350]

C. e. CG--CGGCGG CCTGGCGAAG GCATAAAGCA GTTGATCGCG CCACACCTTA  [400]
C. g. ..AG...... ...T.T.... C..G...... .......A.. ......T.G.  [400]

C. e. CAGGTGCGAT CATACCAGCA CTAATGCACC GGATCCCATC AGAACTCCGC  [450]
C. g. .......... .......... .......... .......... ..........  [450]

C. e. AGTTAAGCGT GCTTGGGCGA GAGTAGTACT AGGATGGGTG ACCTCCCGGG  [500]
C. g. .......... .......... .......... .......... ..........  [500]

C. e. AAGTCCTCGT GTTGCAGCTC CTCCTTTTTT GGTCAGTCAG GTTTGTTCTT  [550]
C. g. .......... .......... .........G C......... .C........  [550]

C. e. TCAAGTGTCG CACACGCGCC GGGTTTTGGA TTATTAGATC GTGC-CGGCG  [600]
C. g. .TG-...G.. .G.......A T.....C.AT .GC.C.A... A.T.T.....  [600]

C. e. ACCCGAGTGG GTCCG---CT CCTCCAAGCC AGATTCTGCC CCGGGCGGCT  [650]
C. g. GTGGAG.C.. .C..AAGA.C G.GG..G.T. G.T...CT.A ..T.......  [650]

C. e. CCGATCCCTG TCGTCGTCGG GCTGGCAGCA AAAGCTCGGA AAATCAGGAT  [700]
C. g. .......T.. G...T.CT.- --------.. .......CA. ..--.G..CC  [700]

C. e. CCGGAACGGG TTTTTGAGGT CGAAATCGAC GTCAACCGGG CTCGCCATGG  [750]
C. g. G......A.. ...C..G.C. ...GG..... C.....A.C. .GAA..C...  [750]

C. e. TTGGGACGTC GACGGGCTCG AGACGAGCTT TCCAACGGTA GCTCCCTCGC  [800]
C. g. ........G. .G.......T G...A..... ....GA..CG C.....GG..  [800]

C. e. CTCGGGATTC AACCCGGATG AAAAGTTACG GCCGTCCGAA GCGAGCACGA  [850]
C. g. ....A..... .T......CA G.......T. AT.A.TT... ...C..G..G  [850]

C. e. GACGGCTCGG CGAAGCCTTA AGGCAGTTGA TCGTGCCACA CTTGACAGGT  [900]
C. g. --...TCTTC .......A.. .AC...AGT. ...C...... ..........  [900]

C. e. GCGATCATAC GAACACTAAT GCATCGGATC CCATCAGAAC TCCGCAGTTA  [950]
C. g. .......... .......... .......... .......... ..........  [950]

C. e. AGCG [954]
C. g. .... [954]          Identical=. Missing=? Indel=-
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Figure 1. Alignment of the major fragments amplified from the 5S rDNA repeats of *Canavalia gladiata* (C. g.) and *Canavalia ensiformis* (C. e.). The entire 120-bp 5S rRNA gene and the 40 and 58 bp of the gene flanking the 5' and 3' ends are enclosed in a box; the intragenic promoter motifs are underlined.

the fragments. The minor fragments (459 bp and 457 bp amplified from *C. gladiata* and *C. ensiformis*, respectively) included the entire 361 bp NTS (in *C. gladiata*) or 359 bp NTS (in *C. ensiformis*). The major fragments (940 bp and 948 bp amplified from *C. gladiata* and *C. ensiformis*, respectively) consisted of two NTS regions separated by the whole gene sequence. The major fragments were deposited in the GenBank

database (accession numbers: KU230029.1 and KU230030.1). The 5' and 3' end NTS regions of the major fragment from *C. gladiata* were both 361 bp but differed in nucleotide composition (variable sites: 35/361; G + C contents: 62.9% and 60.3%, respectively). The 5' and 3' end NTS regions of the major fragment from *C. ensiformis* differed in length (359 bp and 371 bp, respectively) and in nucleotide composition (variable sites: 100/375; G + C contents: 62.4% and 59.1%, respectively). There was a lower level of sequence identity (variable sites: 145/736; identity value: 80.3%) between *C. ensiformis* and *C. gladiata* in terms of the 5' and 3' end NTS regions of their major fragments. The 5S rRNA genes consist of a conserved 120-bp sequence starting with AGG and ending with TCC. According to the BLAST site of the NCBI database, this configuration is almost identical to those of *Vigna angularis* (Willdenow, 1800) Ohwi & H.Ohashi, 1969, *Vigna radiata* (Linnaeus, 1753) R. Wilczek, 1954, *Lupinus luteus* Linnaeus, 1753, *Glycine max* (Linnaeus, 1753) Merrill, 1917 and other Fabaceae species (Gottlob-McHugh et al. 1990, Nuc et al. 1993, Sakai et al. 2015). An intragenic promoter composed of an A-box, an Intermediate Element (IE), and a C-box was identified (Fig. 1) by comparing the 5S rDNA gene sequences of the two *Canavalia* species with that of *Arabidopsis thaliana* (Linnaeus, 1753) Heynhold, 1842 (Cloix et al. 2003).

General karyotype features

Representative mitotic chromosomes of C. *gladiata* and *C. ensiformis* are shown in Figure 2. The chromosome measurements for both species are given in Table 1. Idiograms displaying the chromosome measurements, position and size of the CPD bands, rDNA-FISH signals, and sGISH signals are illustrated in Figure 3.

Both *C. gladiata* and *C. ensiformis* have a diploid chromosome number 2n = 22. The mitotic metaphase chromosomes are rather small. The TCL for *C. gladiata* and *C. ensiformis* are 40.46 ± 1.03 μm and 34.06 ± 3.87 μm, respectively. The individual metaphase chromosomes ranged from 4.72-2.63 μm long in *C. gladiata*, and from 4.21-2.43 μm long in *C. ensiformis*.

Both species have karyotypes composed of metacentric (m) chromosomes only (Table 1; Fig. 3). Chromosome pairs 6 and 7 have satellites with secondary constrictions (SC) located at the interstitial and proximal positions of the short arms, respectively (Fig. 2a, c, i, s, j, l, u). The karyotypes were therefore formulated as 2n = 22 = 18m + 4m-SAT. In most prometaphase (images not shown) and some metaphase cells, the satellites were visualized separately from the rest of the chromosomes with the SC stretched (Fig. 2a, i). At metaphase, the SC of pair 7 in *C. gladiata* stretched more frequently than did that in *C. ensiformis*. Six asymmetry indices, CI, A1, A2, As K%, AI, and the Stebbins' category, are 42.78±2.92, 0.25, 0.18, 57.04, 1.23, and 1A for *C. gladiata*, and 43.31±3.66, 0.23, 0.19, 56.50, 1.63, and 1A for *C. ensiformis*. These data indicate that both karyotypes are similar and symmetric; however, based on AI, the karyotype of *C. ensiformis* is slightly asymmetrical relative to that of *C. gladiata*.

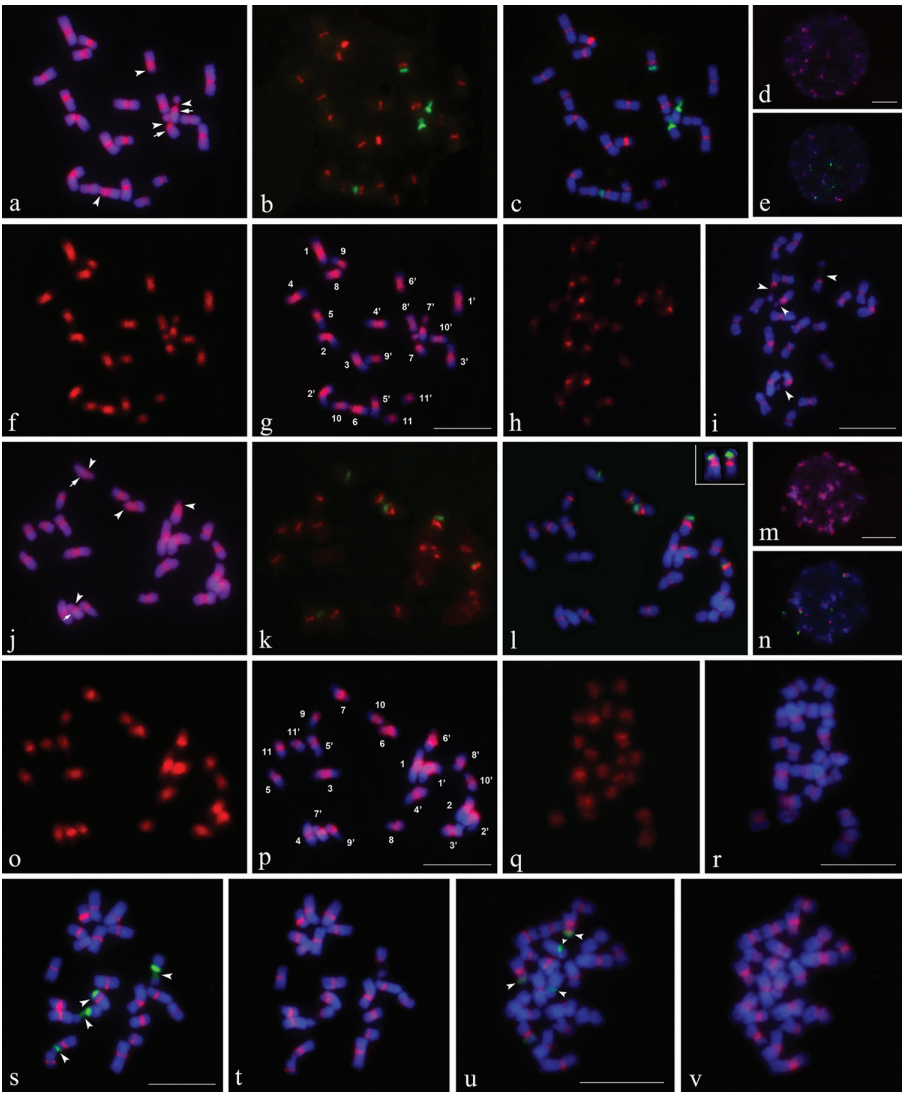

Figure 2. Mitotic chromosomes (except for **d, e, m, n**) and interphase nuclei (**d, e, m** and **n**) of *Canavalia gladiata* (**a–i, s, t**) and *Canavalia ensiformis* (**j–r, u, v**) after sequential CPD staining and *in situ* hybridization. **a, d, j, m** CPD-stained chromosomes and interphase nuclei. **c, e, l, n, s, u** Chromosomes and interphase nuclei showing 5S (red) and 45S (green) rDNA signals produced by digoxigenin-labeled 5S rDNA and biotin-labeled 45S rDNA probes. **b** and **k** 5S and 45S rDNA signals only. **f** and **o** Signals produced by digoxigenin-labeled total genomic DNA of their own, **g** and **p** Chromosomes with sGISH signals. **h** and **q** Signals produced by digoxigenin-labeled total genomic DNA probes from other species. **i** and **r** Chromosomes with cGISH signals. **t** and **v** FISH of digoxigenin-labeled 5S rDNA repeats cloned from *C. gladiata* and *C. ensiformis* to same spreads shown in **s** and **u**, respectively. Arrows in **a** and **j** indicate positions of pair 7 centromeres. Arrowheads in **a, i, j, s** and **u** indicate distinguishable secondary constrictions (SC). Chromosome numbers in **g** and **p** are designated by karyotyping. Chromosomes in upper right corner of **l** are pair 6 from another spread showing proximal 5S rDNA loci on short arms. Chromosomes were counterstained using DAPI (blue). Bars = 10 μm.

Table 1. Chromosome measurements of *Canavalia gladiata* (*C.g.*) and *Canavalia ensiformis* (*C.e.*) obtained from five metaphases per species.

Species	Chr. No.	Relative length (%)			Arm ratio ± SD	Type	Centromeric CPD band size‡ ± SD	sGISH signal size‡		
		Short arm ± SD	Long arm ± SD	Total ± SD				Short arm ± SD	Long arm ± SD	Total ± SD
C. g.	1	5.25 ± 0.23	6.70 ± 0.24	11.95 ± 0.41	1.28 ± 0.05	m	1.53 ± 0.27	3.19 ± 0.19	4.17 ± 0.13	7.35 ± 0.22
	2	4.43 ± 0.10	6.39 ± 0.36	10.82 ± 0.31	1.44 ± 0.10	m	1.93 ± 0.15	3.07 ± 0.33	2.96 ± 0.19	6.03 ± 0.38
	3	4.72 ± 0.37	5.69 ± 0.19	10.40 ± 0.28	1.21 ± 0.12	m	1.63 ± 0.17	1.44 ± 0.28	3.56 ± 0.13	5.00 ± 0.28
	4	4.63 ± 0.20	5.57 ± 0.17	10.20 ± 0.17	1.21 ± 0.08	m	1.67 ± 0.14	3.19 ± 0.22	2.50 ± 0.15	5.69 ± 0.09
	5	3.61 ± 0.08	5.53 ± 0.09	9.15 ± 0.16	1.53 ± 0.02	m	1.76 ± 0.19	3.61 ± 0.08	1.94 ± 0.24	5.55 ± 0.31
	6	3.71 ± 0.46	5.11 ± 0.22	8.83 ± 0.61	1.39 ± 0.16	m†	1.54 ± 0.10	3.71 ± 0.46	2.79 ± 0.26	6.50 ± 0.67
	7	4.14 ± 0.20	4.50 ± 0.26	8.64 ± 0.38	1.09 ± 0.06	m†	1.14 ±0.18	4.14 ± 0.20	2.13 ± 0.32	6.27 ± 0.50
	8	3.42 ± 0.25	4.68 ± 0.12	8.11 ± 0.21	1.38 ± 0.13	m	1.93 ± 0.27	2.33 ± 0.23	3.01 ± 0.14	5.34 ± 0.31
	9	3.20 ± 0.08	4.53 ± 0.20	7.73 ± 0.18	1.42 ± 0.08	m	1.79 ± 0.11	3.20 ± 0.08	1.77 ± 0.21	4.97 ± 0.25
	10	3.39 ± 0.10	4.31 ± 0.29	7.70 ± 0.38	1.27 ± 0.06	m	1.36 ± 0.16	1.62 ± 0.28	2.55 ± 0.32	4.18 ± 0.31
	11	2.44 ± 0.16	4.03 ± 0.21	6.47 ± 0.27	1.65 ± 0.13	m	1.32 ± 0.12	2.43 ± 0.18	1.73 ± 0.17	4.15 ± 0.33
	Total	42.96 ± 0.51	57.04 ± 0.51	100			17.59 ± 1.13	31.93 ± 0.33	29.11 ± 0.32	61.04 ± 0.19
C. e.	1	5.58 ± 0.23	7.02 ± 0.38	12.60 ± 0.18	1.26 ± 0.11	m	2.54 ±0.57	3.18 ± 0.17	3.99 ±0.29	7.17 ±0.36
	2	4.43 ± 0.18	6.62 ± 0.28	11.05 ± 0.38	1.50 ± 0.07	m	2.32 ±0.63	2.53 ± 0.29	3.14 ±0.20	5.66 ±0.40
	3	5.02 ± 0.16	5.33 ± 0.60	10.35 ± 0.51	1.06 ± 0.14	m	2.14 ±0.68	3.77 ± 0.24	2.32 ±0.11	6.10 ±0.29
	4	4.68 ± 0.16	5.35 ± 0.43	10.03 ± 0.53	1.14 ± 0.08	m	1.93 ±0.40	1.67 ± 0.21	3.57 ±0.19	5.24 ±0.37
	5	3.49 ± 0.19	5.36 ± 0.28	8.85 ± 0.29	1.54 ± 0.13	m	1.80 ±0.23	2.12 ± 0.17	2.13 ±0.32	4.25 ±0.31
	6	3.96 ± 0.29	4.80 ± 0.25	8.76 ± 0.46	1.22 ± 0.08	m†	2.08 ±0.51	3.96 ± 0.29	2.87 ±0.24	6.83 ±0.31
	7	3.65 ± 0.25	4.46 ± 0.16	8.11 ± 0.15	1.23 ± 0.13	m†	1.45±0.18	3.65 ± 0.25	2.48 ±0.55	6.13 ±0.57
	8	3.76 ± 0.25	4.25 ± 0.28	8.01 ± 0.41	1.13 ± 0.10	m	1.88 ±0.33	1.65 ± 0.22	2.73 ±0.15	4.38 ±0.34
	9	2.99 ± 0.23	4.73 ± 0.32	7.72 ± 0.23	1.59 ± 0.22	m	1.76 ±0.20	2.99 ± 0.23	1.81 ±0.19	4.80 ±0.35
	10	3.35 ± 0.24	4.34 ± 0.22	7.69 ± 0.44	1.30 ± 0.05	m	1.67 ±0.18	2.06 ± 0.15	2.56 ±0.26	4.62 ±0.37
	11	2.59 ± 0.27	4.23 ± 0.19	6.82 ± 0.44	1.64 ±0.12	m	1.68 ±0.08	2.59 ± 0.27	1.76 ±0.38	4.35 ±0.61
	Total	43.50 ± 0.76	56.50 ± 0.76	100			21.24 ± 3.11	30.18 ± 1.36	29.35 ± 2.27	59.53 ± 3.50

SD, standard deviation. m, metacentric. †satellite chromosome (satellite length was included in chromosome length but secondary constriction length was excluded). ‡ % of band (signal) size in relation to the karyotype length.

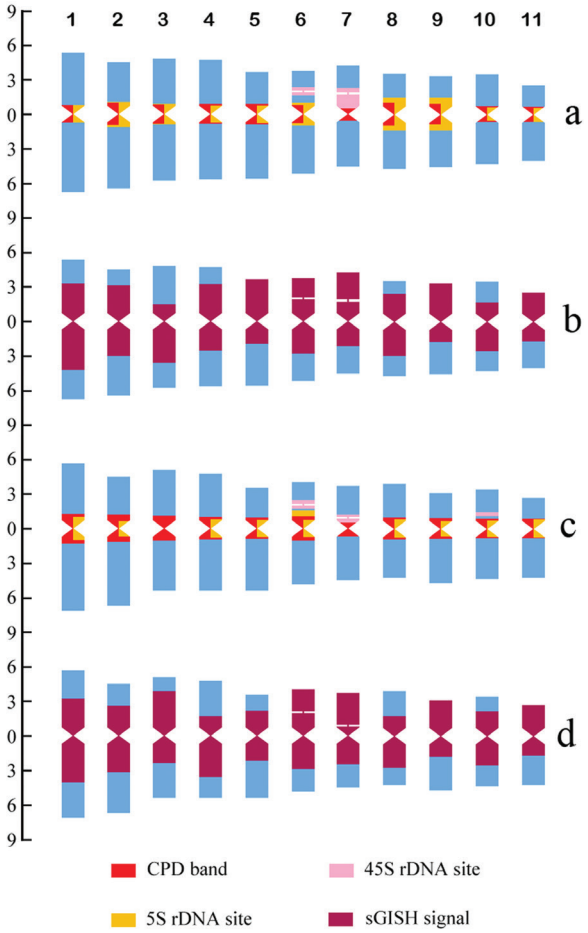

Figure 3. Idiograms of *Canavalia gladiata* (**a, b**) and *C. ensiformis* (**c, d**). **a** and **c** are idiograms displaying chromosome measurements and position and size of fluorochrome bands and rDNA FISH signals, **b** and **d** are idiograms displaying chromosome measurements and size and distribution of sGISH signals. Ordinate scale on left indicates relative chromosome length (% of haploid complement). The numbers above panel **a** are chromosome numbers.

CPD banding patterns

CPD staining revealed that both species had similar fluorochrome banding patterns. The centromeric regions of all chromosome pairs and the 45S rDNA sites demonstrated by sequential rDNA-FISH appeared as red CPD bands (Fig. 2a, j). The pair 6 rDNA CPD bands did not significantly differ in size and intensity between the two species. Nevertheless, the pair 7 rDNA CPD bands of *C. gladiata* were larger and more intense than those of *C. ensiformis.* The pair 10 rDNA CPD bands of *C. ensiformis* were juxtaposed with the centromeric CPD bands. The primary constrictions of pair 7 in both species were not as obvious as those of other pairs and were assumed to be

adjacent to the proximal regions of the rDNA CPD bands. They displayed small, weak CPD bands (Fig. 2a, j). The size of the centromeric CPD bands was expressed as a percentage of the karyotype length and ranged from 1.14–1.93% in *C. gladiata*, and 1.45-2.54% in *C. ensiformis*. The centromeric bands of *C. gladiata* occupied 17.59% and those of *C. ensiformis* took up 21.24% of the total karyotype length (Table 1; Fig. 3). Up to 24 red-fluorescing heterochromatin blocks of different sizes were observed in the CPD-stained interphase nuclei of both species (Fig. 2d, m).

rDNA FISH patterns

FISH analyses of the 5S and 45S rDNA probes to the CPD-stained mitotic chromosomes and interphase nuclei are presented in Fig. 2. Ten 5S rDNA loci were detected in both species. In *C. gladiata*, the 5S signals were observed in the centromeres of all but the seventh chromosome pair and were strongest for pair 9 and weakest for pair 10 (Fig. 2b, c, s). In *C. ensiformis,* 5S signals were found in the centromeres of all but the 3rd and 7th pairs, and the proximal regions of the short arms of pair 6 (*di* = 32.07%). There were no significant differences in intensity (Fig. 2k, l, u). In interphase cells, the 5S signals were all co-localized with the CPD-banded heterochromatin blocks (Fig. 2e, n). Two and three loci for 45S rDNA were detected in *C. gladiata* and *C. ensiformis*, respectively. Two pairs of 45S signals associated with the SC of the satellite chromosome pairs 6 and 7 were detected in both species (*di* = 54.32% for pair 6 and 38.67% for pair 7 in *C. gladiata*; *di* = 50.57% for pair 6 and 26.14% for pair 7 in *C. ensiformis*). These correspond to their respective CPD bands in both size and intensity (Fig. 2a, b, c, j, k, l, s, u). In *C. ensiformis*, a minor 45S locus was observed in the proximal regions of the short arms of pair 10 (*di* = 29.05%; Fig. 2k, l, u). The 45S signals of pair 6 for both *C. gladiata* and *C. ensiformis* were similar in intensity. The 45S signals of pair 7 in *C. gladiata* were much stronger than those in *C. ensiformis* (Fig. 2b, c, k, l, s, u). At interphase, dispersed 45S signals were found. These consisted of four or six strongly fluorescing knobs with varying numbers of weakly fluorescing spots emanating from them (Fig. 2e, n).

FISH performed on mitotic chromosomes using the cloned major 5S rDNA fragment probe generated signals in the regions corresponding to the 5S signals from pTa794 and in the centromeres wherein no signal was generated using pTa794 (Fig. 2t, v). The signals from the cloned major 5S rDNA fragments were slightly larger and stronger than those produced by pTa794 (Fig. 2s, t, u, v).

Self-GISH signal patterns

The chromosomal distribution patterns of repetitive DNA sequences were investigated using self-GISH. Distinct sGISH signal patterns were generated in both species and they were largely similar to each other (Figs 2f, g, o, p; 3b, d). sGISH signals appeared

on each chromosome in the complement and accounted for 61.04% of the total karyotype length in *C. gladiata* and 59.53% in *C. ensiformis* (Table 1). The size of the sGISH signal in each chromosome pair was expressed as a percentage of the karyotype length. It varied from 4.15–7.35% in *C. gladiata* and from 4.25–7.17% in *C. ensiformis* (Table 1; Fig. 3b, d). The signals were distributed in all pericentromeric regions, the proximal regions of some chromosome arms, and entire short arms of certain chromosome pairs. The genomic probe intensely labeled the 45S rDNA sites in both species. The distal regions of most chromosome arms (17–18 arms of the haploid complement) had no fluorescence. In particular, the size and location of the sGISH signal of each chromosome pair are unique and, along with the measurements and rDNA-FISH signals, enable each metaphase chromosome to be identified accurately (Figs 2g, p; 3b, d). In *C. gladiata*, the short arms of pairs 5, 6, 7, 9, and 11 were entirely labeled. The signal sizes on both the short and long arms of pairs 1, 2, 6, 8, and 11 were similar. The signal sizes on the long arms of pairs 3 and 10 were much larger than those on their short arms. The signal sizes on the long arms of pairs 4, 5, 7, and 9 were much lower than those on their short arms (Figs 2g; 3b). In *C. ensiformis*, the signal patterns of pairs 1, 2, 6, 7, 9, and 11 resembled the same ones in *C. gladiata.* The signal patterns of pairs 3 and 4 in *C. ensiformis* resembled those of pairs 4 and 3 of *C. gladiata*, respectively. The short arm of pair 5 was not entirely labeled. The distal regions lacked any fluorescent signal. In contrast, for *C. ensiformis,* the signal of the short arm of pair 8 decreased and that of pair 10 increased relative to those in *C. gladiata* (Figs 2p; 3d). For both species, the total amounts of sGISH signal in both short and long arms of the complement were nearly the same (Table 1).

Comparative GISH signal patterns

cGISH was employed to probe the gDNA signals on the metaphase chromosomes of another species (Fig. 2h, i, q, r) to reveal the homology of repetitive DNA sequences between the two species. On the metaphase chromosomes of *C. gladiata*, the gDNA of *C. ensiformis* generated signals in all pericentromeric regions and 45S rDNA sites. Most centromeres and the 45S rDNA sites of pair 7 were strongly labeled compared with other regions (Fig. 2h, i). In *C. ensiformis*, cGISH with *C. gladiata* gDNA also produced strong signals in all pericentromeric regions and 45S rDNA sites. The highest intensity was observed at the centromeres (Fig. 2q, r).

Discussion

Characteristics of the two *Canavalia* genomes

In this study, detailed karyotypes of *C. gladiata* and *C. ensiformis* were established using a combination of chromosome measurements, CPD bands, rDNA-FISH signals, and

sGISH signals. The karyotypes provided the first molecular cytogenetic characterization of the two cultivated *Canavalia* species. The sGISH and rDNA-FISH signals were effective cytogenetic markers enabling unambiguous identificaion of individual chromosomes in both species.

The data revealed that the karyotypes of both *C. gladiata* and *C. ensiformis* are quite symmetrical. The karyotype of *C. ensiformis* has not been reported previously. The karyotype of *C. gladiata* in the present study shows more symmetry and differs from those described by Li (1989), Bairiganjan and Patnaik (1989), and Chen (2003). Discrepancies in karyotype formula were probably due to differences in the material analyzed and difficulties in identifying chromosomes using classical staining techniques.

The rDNA-FISH revealed that there are a substantial number of 5S rDNA loci located in the centromeres in both species. There should be 5S rDNA repeats in all centromeres in both species because FISH using the cloned major 5S rDNA fragment generated weak signals in the centromeres wherein no signal was detected by pTa794. The copy number of 5S rDNA repeats within the centromeres of pair 7 (both species) and pair 3 of *C. ensiformis* was probably too low to be detected by FISH using the exogenous 5S rDNA probe. Centromeric 5S rDNA arrays have seldom been detected in plants by FISH. One to several centromeric 5S loci have only been reported for two *Grindelia* (Willdenow, 1807) species (Baeza and Schrader 2005), *Podophyllum hexandrum* Royle, 1834 (Nag and Rajkumar 2011), *Paphiopedilum* Pfitzer, 1886 (Lan and Albert 2011), two *Alstroemeria* (Linnaeus, 1762) species (Chacón et al. 2012), and *Vigna aconitifolia* (Jacquin, 1768) Maréchal, 1969 (She et al. 2015). The centromeric regions in plants, including Phaseoleae species, consist of different satellite DNA families and transposable elements (Jiang et al. 2003, Tek et al. 2010, Iwata et al. 2013). The 5S rDNA signals may not actually be located in the functional regions of the centromeres even though they seemed to coincide exactly with them. It is worth verifying whether 5S rDNA repeats participate in centromere function using immunofluorescence and chromatin immunoprecipitation (ChIP)-based assays (Tek et al. 2010, Iwata et al. 2013).

Another prominent feature of the two *Canavalia* genomes was the non-rDNA GC-rich heterochromatin in all centromeres (highlighted by CPD staining) (She et al. 2006). Centromeric, pericentromeric, or proximal non-rDNA GC-rich heterochromatin have been observed in many Phaseoleae, including *Psophocarpus tetragonolobus* A. P. de Candolle, 1825 (Chaowen et al. 2004), four cultivated *Phaseolus* (Linnaeus, 1754) species (Bonifácio et al. 2012), seven cultivated *Vigna* (Savi, 1824) species (She et al. 2015), Lablab purpureus (Linnaeus, 1763) Sweet, 1826 (She and Jiang 2015), and *Crotalaria* (Linnaeus, 1753) species from two sections of the tribe Crotalarieae (Mondin and Aguiar-Perecin 2011) which is a branch of the Genistoid clade (LPWG 2013). A recent multilocus phylogenetic analysis reestablished the tribe Diocleae as a branch of the Phaseoloid (Millettioid) clade, which includes the *Canavalia* and two other clades (Queiroz et al. 2015). It is therefore proposed that the presence of (peri)centromeric GC-rich heterochromatin is an ancestral characteristic existing before the origin of Phaseoloid (LPWG 2013). In the two *Canavalia* species, however, most centromeric CPD

bands should arise when 5S rDNA repeats intersperse with other GC-rich repeats. All but one centromeric CPD band in *C. gladiata* and two in *C. ensiformis* were co-localized with 5S rDNA arrays. Nevertheless, they did not completely correspond in size to the 5S signals. The sequence analysis revealed the NTS of 5S rDNA repeats of both species was GC-rich. GC-rich regions co-localized with 5S rDNA sites have also been observed in other plants (e.g. Zoldos et al. 1999, Hamon et al. 2009, She et al. 2015).

The sGISH experiments revealed a distinct distribution of repetitive DNA sequences on the chromosomes of the two *Canavalia* species. sGISH data obtained from many plants showed that the chromosomal distribution of repetitive sequences is often non-uniform and forms clusters within heterochromatin blocks, and two different sGISH patterns may occur depending on the genome size of the species (She et al. 2007). In plants with small, compact genomes, the hybridization signals concentrate mainly in (peri)centromeric or proximal regions, heterochromatic arms, and 45S rDNA sites (Falistocco et al. 2002, Maluszynska and Hasterok 2005, She et al. 2007, Wolny and Hasterok 2009, Falistocco and Marconi 2013, She et al. 2015). In plants with large genomes, the entire chromosome length is densely labeled with strongly and weakly labeled regions alternate, or with enhanced signals located in C-band regions (She et al. 2007, Zhou et al. 2008). The repetitive sequence distribution patterns in *C. gladiata* and *C. ensiformis* generally resemble those of small plant genomes reported previously but had their own unique characteristics not reported elsewhere. The repetitive sequences are distributed asymmetrically on both sides of the centromeres, unequally between chromosome pairs, but evenly between the short and long arms in the complement.

Similarities and differentiation between the two *Canavalia* genomes

The molecular cytogenetic data obtained in this study revealed a high degree of similarity in genome organization between the two *Canavalia* species. This result confirms the evolutionary closeness between *C. gladiata* and *C. ensiformis* which was previously inferred from morphological and seed protein comparisons (Smartt 1990) and molecular phylogenetic analysis (Snak et al. 2016). Both species had the same karyotype formula and similar karyotype indices. The chromosome arrangements in the complement did not differ except for the exchange of pairs 3 and 4. The distributions of their centromeric CPD bands were similar. Most of their chromosome pairs had similar sGISH signal patterns. The 45S loci on pairs 6 and 7 and the centromeric 5S rDNA loci of nine chromosome pairs were conserved. The seventh chromosome pair lacked 5S rDNA signals. Extensive cross-hybridization and highly similar signal patterns resulted from reciprocal cGISH, which indicates high repetitive DNA homology and reflects their close phylogenetic relationships (Maluszynska and Hasterok 2005, She et al. 2015, Zhang et al. 2015).

The data also revealed distinct differences between the two genomes. The genome size of *C. ensiformis* was nearly one-sixth less than that of *C. gladiata* based on their TCL (Levin 2002). Rodrigues and Torne (1990) reported that the TCL of *C. ensiformis* was

only 70.55% that of *C. gladiata*. The karyotype of *C. ensiformis* was more asymmetrical than that of *C. gladiata*. *C. ensiformis* with its annual life form and a more restained and bushier growth habit is considered to be more advanced in evolution than *C. gladiata*, which is closer to the wild species with its perennial life form and twining growth habit (Smartt 1990). Our karyotypic data support this opinion since a symmetrical karyotype is considered characteristic of more primitive species (Stebbins 1971). Furthermore, the karyotypic differences between the two species coincide with a karyotype evolutionary pattern in which increasing specialization is accompanied by genome size reduction, particularly where the specialization involves a shift to an annual habit or a shorter growing season. This downsizing results in an increase in karyotype asymmetry (Levin 2002). Nevertheless, detailed karyotyping revealed that the significant genome size contraction in *C. ensiformis* did not significantly change its karyotype morphology and complement sGISH signal proportion and distribution relative to those of *C. gladiata*. Therefore, the karyotypic comparison between the two species corroborates the increasing karyotype asymmetry hypothesis proposed by Levin (2002). This theory proposed that genome contraction is achieved by an equal reduction in the amount of DNA per chromosome regardless of chromosome size. This mechanism increases asymmetry.

Compared to *C. gladiata*, *C. ensiformis* gained an extra proximal 45S rDNA locus and a non-centromeric 5S rDNA locus but lost a centromeric 5S rDNA locus. Based on the signal intensity (Maluszynska and Heslop-Harrison 1991), the number of 45S rDNA repeats in pair 7 and 5S rDNA repeats in pairs 9 and 10 of *C. ensiformis* changed significantly. Differentiation among species in the chromosomal organization of rDNA clusters has been found in many genera and correlates with chromosome evolution during speciation (e.g. Moscone et al. 1999, 2007, Datson and Murray 2006, Chung et al. 2008, Weiss-Schneeweiss et al. 2008, Morales et al. 2012, She et al. 2015). As mentioned above, *C. gladiata* is closer to wild species than is *C. ensiformis*. Therefore, the rDNA pattern of *C. ensiformis* may have evolved from that of *C. gladiata*. The proximal 45S rDNA locus might have originated from the transposition of the SC-associated 45S rDNA cluster (Datson and Murray 2006, Chung et al. 2008). The proximal 5S locus on the short arms of pair 6 may have arisen from an inversion of the segment bearing part of the centromeric 5S rDNA (Moscone et al. 2007, Weiss-Schneeweiss et al. 2008). The disappearance of the 5S rDNA signal at the centromeres of pair 3 may have come from the significant reduction of 5S rDNA repeats in this region (Chung et al. 2008).

sGISH revealed that the distribution of repetitive sequences on pairs 5, 8, and 10, differed significantly between the two species. This fact suggests that *C. ensiformis* lost repetitive DNAs in some chromosomal regions and/or its chromosomes were rearranged during its evolution. Sequence analysis of 5S rDNA repeats revealed a lower level of NTS sequence identity between the species, indicating that their genomic sequences were clearly differentiated (Liu et al. 2003). The percentage of centromeric CPD bands in the complement of *C. ensiformis* was one-fifth (20%) greater than that of *C. gladiata*, reflecting an increase of the proportion of GC-rich heterochromatin in *C. ensiformis* (She et al. 2006).

Conclusions

Individual chromosomes of both *C. gladiata* and *C. ensiformis* can be accurately identified by sGISH and rDNA-FISH signals.

Both *C. gladiata* and *C. ensiformis* genomes have particular characteristics including existence of non-rDNA GC-rich heterochromatin at all centromeres and 5S rDNA loci at the vast majority of centromeres, and a unique chromosomal distribution of repetitive DNA sequences.

Molecular cytogenetic comparison revealed both basic similarities and distinct differences in genome organization between *C. gladiata* and *C. ensiformis*, providing insights into the evolutionary relationships between them.

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REVIEW ARTICLE

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Comparative analysis of chromosomal localization of ribosomal and telomeric DNA markers in three species of Pyrgomorphidae grasshoppers

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Abstract

The karyotypes of three species of Pyrgomorphidae grasshoppers were studied: *Zonocerus elegans* (Thunberg, 1815), *Pyrgomorpha guentheri* (Burr, 1899) and *Atractomorpha lata* (Mochulsky, 1866). Data on karyotypes of *P. guentheri* and *Z. elegans* are reported here for the first time. All species have karyotypes consisting of 19 acrocentric chromosomes in males and 20 acrocentric chromosomes in females (2n♂=19, NF=19; 2n♀=20, NF=20) and X0/XX sex determination system. A comparative analysis of the localization of C-heterochromatin, clusters of ribosomal DNA, and telomere repeats revealed inter-species diversity in these cytogenetic markers. These differences indicate that the karyotype divergence in the species studied is not associated with structural chromosome rearrangements, but with the evolution of repeated DNA sequences.

Keywords

Pyrgomorphidae grasshoppers, karyotype, C-banding, FISH, 28S rDNA, telomeric DNA

Introduction

Orthoptera is undoubtedly one of the most well cytogenetically studied groups of insects. Even at an early stage of comparative cytogenetics, they became convenient research models for analysis of mitotic and meiotic chromosomes. It was through working on Orthoptera that Robertson (1916) established the main tendencies in insect karyotype evolution through centric fusion of chromosomes, Darlington (1931, 1932) described meiosis in detail, and White (1968, 1973) proposed the chromosome speciation hypothesis.

However, the karyotypic features of various Orthoptera groups have been studied extremely unevenly. Among Acridoidea and Pyrgomorphoidea, only the family Acrididae can be considered as well studied, whereas the karyotypes of Pyrgomorphidae, Pamphagidae, Lathiceridae, Lentulidae and some other families remain poorly investigated or not studied at all. Analysis of chromosome sets within such Orthoptera groups, which have never been studied before, in conjunction with the use of new techniques for chromosome research, may therefore potentially lead to many new insights. As an example, using molecular cytogenetic methods, in-depth research of the family Pamphagidae has recently revealed new evolutionary pathways of autosomes and sex chromosomes previously unknown in this family (Bugrov et al. 2016, Jetybayev et al. 2017).

The basal chromosome set of the family Pyrgomorphidae (superfamily Pyrgomorphoidea) coincides with that of the family Pamphagidae (superfamily Acridoidea) and contains 19 acrocentric chromosomes in males, 20 in females (sex determination X0/XX) (White 1973, Hewitt 1979). In this regard, these two families with FN=19♂/20♂ differ from other Acridoidea species, the basal karyotype of which contains 23 acrocentric chromosomes in males, 24 in females (sex determination X0/XX FN=23♂/24♀). The morphological similarity of the modal chromosome set in Pamphagidae and Pyrgomorphidae was noted a long time ago (White 1973, Hewitt 1979); however, the question as to whether this similarity represents a phylogenetic signal is still unknown. This is partially related to the poor degree of karyological study of Pyrgomorphidae grasshoppers. The karyotypes of only about 30 species are known from tropical and subtropical regions of the Old World (Makino 1951, Sannomiya 1973; Nankivell 1976, John and King 1983, Fossey et al. 1989, Williams and Ogunbiyi 1995, Seino et al. 2013, Seino and Dongmo 2015). The vast majority of species have a 19-chromosome karyotype, but a few species have been shown to have a different karyotype, resulting from one, two or three Robertsonian translocations (White 1973, Fossey et al. 1989). Moreover, only in a few species the C-heterochromatin localization has been studied (*Atractomorpha similis*, *A. hypoestes*, *A. australis*; *Pyrgomorpha conica*) (Nankivell 1976, John and King 1983, Suja et al. 1993).

Molecular cytogenetic studies were previously performed for only one species of Pyrgomorphidae – *Pyrgomorpha conica* (Suja et al. 1993, López-Fernández et al. 2004, 2006).

The aim of the present study, therefore, is to reveal new features of chromosome sets in as-yet unstudied species of Pyromorphidae grasshoppers. We used standard cytoge-

netic techniques, as well as molecular-cytogenetic methods, to find additional markers of linear chromosome differentiation. The fluorescence *in situ* hybridization (FISH) method was employed to localize functionally important regions in autosomes and the sex chromosomes, containing clusters of ribosomal DNA and telomeric $(TTAGG)_n$ repeats. The choice of these molecular markers was prompted by an awareness of their important functional role in the genome and chromosome localization of many insects including Pyrgomorphidae grasshoppers (Sahara et al. 1999, López-Fernández et al. 2004, Cabrero and Camacho 2008), and renders the data reported herein suitable for comparative analysis.

Material and methods

Material collection, fixation and C-banding

Three species belonging to Pyrgomorphidae were studied: 1) *Zonocerus elegans* (Thunberg, 1815) (Phymateini tribe) – six males of this species collected during February and March 2003 in South Africa, in vicinity of Springbok city; 2) *Pyrgomorpha guentheri* (Burr, 1899), (Pyrgomorphini tribe) – five males of this species collected in June 2007 in Armenia; 3) *Atractomorpha lata* (Mochulsky, 1866) (Atractomorphini tribe) – two males of this species collected in August, 2005 on Ishigaki island (Ryukyu Archipelago, Japan).

The collected insects were injected with 0.1% colchicine solution and, after 1.5–2.0 hours, their testes were dissected and placed into 0.9% solution of sodium citrate for 20 minutes, then fixed in 3:1 ethanol:glacial acetic acid for 15 minutes. The fixed material was rinsed and kept in 70% ethanol.

C-banding of the chromosome preparations was performed according to the protocol of Sumner (1972), with minor modifications.

Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization on meiotic chromosomes was carried out according to the protocol of Pinkel (1986) with modifications (Rubtsov et al. 2000, 2002). The rDNA probe was obtained as was described earlier (Jetybayev et al. 2017). The sequences of primers used for 28S rDNA were designed on the basis of consensus sequence of the 28S rRNA gene, obtained by the alignment of rDNA sequences of different species of grasshoppers (gb|AY859546.1, gb| KM853499.1, gb|AY125286.1 and gb|EU414723.1), using the software packages PerlPremier (Marshall 2004) and Mulalin (Corpet 1988) (Table 1). The DNA probe for detection of telomeric repeats $(TTAGG)_n$ in metaphase chromosomes was generated with non-template PCR (Ijdo et al. 1991) with 5'-TAACCTAACCTAACCTAACC-3' and 5'-TTAGGTTAGGTTAGGTTAGG-3' primers according to standard protocol

Table 1. Primers used for 28S rDNA amplification.

Name	Sequence	Amplicon size
28SrDNA1F	5'-TGGACAATTTCACGACCCGTC-3'	600 bp
28SrDNA1R	5'-GCGTTTGGTTCATCCCACAG-3'	
28SrDNA2F	5'-TGAACCAAACGCCGAGTTAAGG-3'	650 bp
28SrDNA2R	5'-ATTCCAGGGAACTCGAACGCTC-3'	
28SrDNA3F	5'-TTCTGCATGAGCGTTCGAGTTC-3'	700 bp
28SrDNA3R	5'-TGGGCAGAAATCACATTGCGTC-3'	

(Sahara et al. 1999). DNA labelling was performed in additional PCR cycles with Tamra-5-dUTP and Fluorescein-12-dUTP (Biosan, Novosibirsk, Russia).

Chromosome counterstaining was preformed after FISH with 4′,6-diamidino-2-phenylindole (DAPI) using Vectashield antifade containing 200 ng/ml DAPI.

Microscope analysis

Microscopic analysis was performed at the Center for Microscopy of Biological Objects (Institute of Cytology and Genetics, Novosibirsk, Russia). Chromosomes were studied with an AxioImager M1 (Zeiss) fluorescence microscope equipped with filter sets #49, #46HE, #43HE (Zeiss) and a ProgRes MF (MetaSystems) CCD camera. The ISIS5 software package (MetaSystems GmbH, Germany) was used for image capture and analysis.

Chromosome nomenclature

The nomenclature suggested for grasshoppers (King and John 1980, Santos et al. 1983, Cabrero et al. 1985) was used in the description of chromosomes, karyotypes and C-banding.

Results

Karyotype

Data on karyotypes of *P. guentheri* and *Z. elegans* are reported for the first time. Karyotype of *A. lata* was described earlier (Makino 1951). The karyotype reference for this species, reported from Cameroon (Seino et al. 2014), requires verification, given that the distribution of this species is restricted to South-East Asia (http://orthoptera.speciesfile.org).

Diploid sets (2n) of chromosomes in all species studied consisted of 19 (♂) and 20 (♀) acrocentric chromosomes. Sex determination was X0 male and XX female. The karyotype structure consists of three large (L_1–L_3), five medium (M_4–M_8) and one

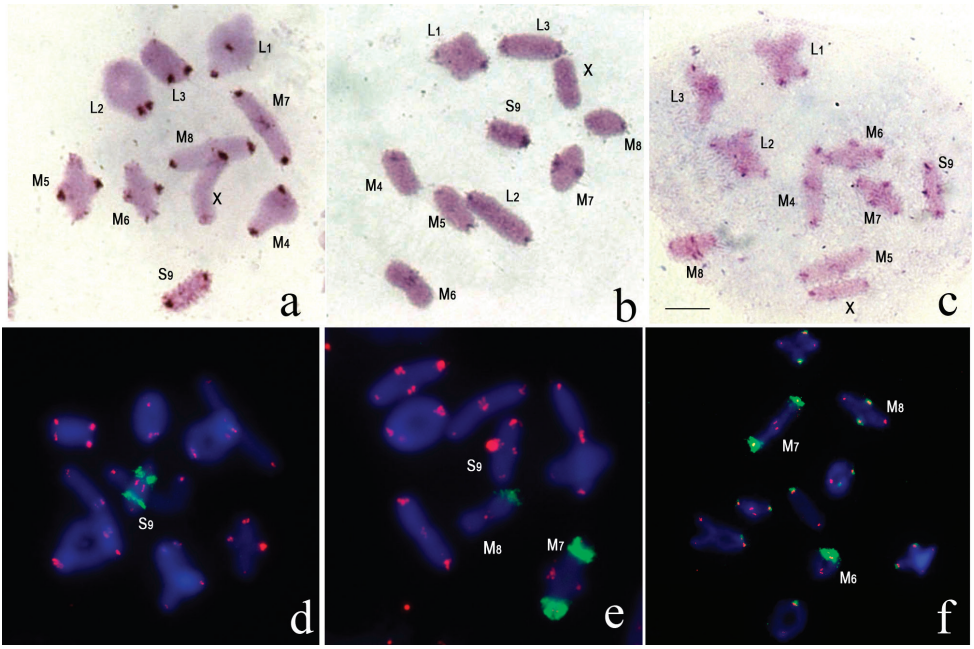

Figure 1. C-banding (**a–c**) and fluorescence *in situ* hybridization of 28S ribosomal DNA (green) and telomere $(TTAGG)_n$ (red) probes (**d–f**) with chromosomes of: **a, d** *Zonocerus elegans*, metaphase I of meiosis **b, e** *Atractomorpha lata,* metaphase I of meiosis **c, f** *Pyrgomorpha guentheri,* metaphase I of meiosis Bar = 5 μm.

small (S_9) pair of autosomes. The fundamental number of chromosome arms (FN) was 19 in male and 20 in female.

The large autosomes of *Z. elegans* and *A. lata* were distinctly different from each other, while the large chromosome pairs (L_1–L_3) of *P. guentheri* and *A. lata* were almost equal in size (Fig. 1a, b, c). The medium and small autosomes varied slightly in size and represented a gradually decreasing size range. All the species studied had a large acrocentric X chromosome, which was almost equal to the L_1 chromosome (Fig. 1a, b, c). At meiotic prophase in *Z. elegans* and *A. lata*, each large bivalent usually formed two, rarely one chiasmata, while medium and small bivalents formed one chiasma (Fig. 1a, b, d, e). In *P. guentheri* each bivalent formed only one chiasma (Fig. 1c, f).

C-banding

In the karyotype of *Zonocerus elegans*, C-banding revealed large paracentromeric C-blocks in all chromosomes of the set. Small terminal C-positive blocks were localized in M_5, M_6, M_7 medium size autosome pairs and the X chromosome. The S_9 autosome is megameric: multiple small C-heterochromatin blocks are located within the whole autosome length (Fig. 1a).

In *Atractomorpha lata*, medium sized pericentric C-blocks were revealed in the L_1–L_3, M_7 and S_9 autosome pairs. The rest of the medium sized autosomes (M_4, M_5, M_6, M_8) and X chromosome had small pericentric C-blocks. In L_1, M_4, M_8 and S_9 bivalents the pericentromeric C-blocks exhibited variation in size in homologous chromosomes. On one of the chromosomes in these bivalents pericentromeric C-block was large, while on the other chromosome it was small (Fig. 1b).

C-banding of *Pyrgomorpha guentheri* chromosomes revealed a medium sized pericentromeric C-block in all autosomes with the exception of the L_3 pair, which had a small block. The pericentromeric C-block on the X chromosome was small. Medium sized terminal C-blocks were found in M_4, M_6, M_7, M_8, S_9 chromosomes (Fig. 1c).

Fluorescence *in situ* hybridization (FISH) of chromosomes with ribosomal and telomeric DNA probes

Analysis of fluorescence *in situ* hybridization of telomeric DNA-probes showed that in all the species studied, telomeric repeats were localized only in terminal areas of all chromosomes. In *Atractomorpha lata* FISH revealed difference in the size of telomeric cluster in a small pair (S_9). Fluorescent signal was significantly stronger on one of the homologous chromosomes in S_9 bivalent (Fig.1e).

FISH of the 28S ribosomal DNA probe revealed interspecific variation of rDNA localization. In *Zonocerus variegatus*, clusters of rDNA were localized in the interstitial region of the S_9 autosome (Fig.1d). In *Atractomorpha lata*, rDNA clusters were observed in pericentromeric regions of two pairs of autosomes (M_7, M_8). In one specimen, in M_8 pair the rDNA cluster was observed only on one of the homologous chromosomes in the bivalent (Fig. 1e). In *Pyrgomorpha guentheri* rDNA clusters were localized in the pericentromeric region of all chromosomes. Most of the rDNA clusters were small, whereas the clusters in the M_6, M_7, and M_8 chromosome pairs were large (Fig. 1f).

Discussion

Comparative analysis of karyotypes of three species of Pyrgomorphidae grasshoppers from the Ethiopian, Mediterranean and East Asian regions confirms that 2n♂=19 (NF=19), 2n♀=20 (NF=20) (X0/XX sex determination) is the basal chromosome set in this group. However, differences from the basal chromosomal set were also observed. Some species exhibit one (*Sphenarium mexicanum*, 2n♂=17), three (*Pyrgomorpha granulata*, 2n♂=13) or four (*Pyrgomorpha rugosa*, 2n♂=11) Robertsonian translocations (White 1973, Fossey et al. 1989). Another variant of non-basal karyotype was described in *Pyrgomorpha* sp. White (1973), referring to his unpublished data, mentions that the 2n=18, XX♀/XY♂ karyotype in this species resulted from centric fusion of acrocentric X-chromosome and acrocentric autosome. In all the cases mentioned above, the fundamental karyotype number remains constant: NF♂=19, NF♀=20.

Searching for new karyotype variants in this poorly studied group holds the potential to turn up interesting findings. For instance, recently, a new model of the Y-chromosome evolution was proposed based on studies in Pamphagidae grasshoppers. It was shown that in Pamphagidae grasshoppers centric fusion of the X chromosome and autosome occurred independently in two phylogenetic branches, and due to further evolution the neo-Y chromosome exhibited different stages of degradation process (Bugrov et al. 2016, Jetybayev et al. 2017).

The Pyrgomorphidae and Pamphagidae both have NF♂=19, NF♀=20, while Acridoidea has NF♂=23, NF♀=24. This gives rise to a question about the monophyly or homoplasy of Pyrgomorphidae and Pamphagidae. However, further detailed analysis of linear chromosome differentiation in these families is needed to shed light on this issue.

The present study revealed the difference in size and localization of C-positive blocks of chromosomes between the species studied. Furthermore, in *A. lata* and *P. guentheri* the difference observed on homologous chromosomes suggests the presence of the polymorphism in population of these species. A high level of interpopulation polymorphism of C-positive regions was previously reported for three Pyrgomorphidae species from Australia, Papua-New Guinea and Indonesia (Nankivell 1976, John and King 1983). Different populations of *Atractomorpha crenaticeps*, *A. similis* and *A. australis* were found to show polymorphism in terms of the size and localization of C-blocks in pericentromeric, interstitial, and telomeric regions in large and medium chromosomes. Furthermore, in some populations of *A. australis* additional arms were found, consisting of very large C-heterochromatin. Later some supernumerary heterochromatic segments in two chromosome pairs were revealed in *Pyrgomorpha conica* (Suja et al. 1993).

Such diversity in terms of the size and localization of C-positive blocks within different species of Pyrgomorphidae grasshoppers indicates that the evolution of repeated DNA sequences plays an important role in the divergence of karyotypes in this group.

However, molecular cytogenetic studies of repetitive sequences in chromosomes of Pyrgomorphidae grasshoppers were carried out only in *Pyrgomorpha conica* (Suja et al. 1993, López-Fernández et al. 2004, 2006). These methods showed that supernumerary heterochromatic segments derived from amplification of rRNA genes (Suja et al. 1993) and telomeric repeats enrich pericentric C-positive blocks (López-Fernández et al. 2006).

The current study represents comparative analysis of localization of 28S rDNA and telomeric $(TTAGG)_n$ sequences in this group. Telomeric repeats exhibited very conservative localization, only in terminal areas of all chromosomes, and no interstitial telomeric sites. This may indicate that the karyotype evolution of these species did not include chromosome structural reorganizations involving terminal regions of chromosomes (for example pericentric inversions). However, interstitial telomeric sequences have previously been reported for Acrididae grasshoppers; such localization of clusters of telomeric DNA may be the result of such chromosomal reorganizations (Jetybayev et al. 2012). The observed polymorphism in the size of the telomeric cluster in *A. lata* correlates with C-block polymorphism in S_9 chromosome. Previously the same kind of

polymorphism in terms of size was reported for *Pyrgomorpha conica* (López-Fernández et al. 2006). The C-blocks consist of amplified repetitive sequences, and sometimes amplification could involve telomeric or rDNA repeats.

Fluorescence hybridization *in situ* (FISH) of the rDNA fragment revealed a consistent pattern of rDNA distribution in chromosomes of the Pyrgomorphidae family. Ribosomal DNA clusters may be found in one pair (S_9 in *Z. elegans*), two pairs (M_7, S_9, in *A. lata*) or in all chromosomes (the pericentric regions of chromosomes in *P. guentheri*). However, in *P. guentheri* most of the rDNA clusters were very small and only clusters on the chromosomes M_7, M_8 and S_9 were significantly larger. This might be the result of a recent expansion of rDNA repeats in pericentric heterochromatin and the newly arisen rDNA clusters may be silent (Suja et al. 1993, Cabrero and Camacho 2008, Jetybayev et al. 2012).

The diversity in the rDNA distribution itself apparently reflects the degree of divergence in the species studied, which belong to different tribes of Pyrgomorphidae. Comparing the patterns of rDNA distribution in the karyotypes of the species studied here with known data on rDNA distribution in karyotypes of other Orthoptera, we may suggest that Pyrgomorphidae are close to the Acrididae family of Orthoptera. In this family, distribution of rDNA is basically limited to one or two pairs of chromosomes in the karyotype (Cabrero and Camacho 2008). In single cases, clusters of rDNA were revealed in the pericentric heterochromatin of all chromosomes in the set (Jetybayev et al. 2012). In contrast to Pyrgomorphidae and Acrididae grasshoppers, multiple localization of rDNA clusters on one chromosome in the Pamphagidae family has been shown (Bugrov et al. 2016, Jetybayev et al. 2017). Perhaps, the revealed differences in the localization of rDNA in Pyrgomorphidae and Acrididae on the one hand, and Pamphagidae on the other hand, may contain a certain phylogenetic signal. However, we still lack enough data, especially for the Pyrgomorphidae family, to approach the problem of the origin of the modal 19-chromosome karyotype of Pyrgomorphidae and Pamphagidae from a molecular-cytogenetic position. Nevertheless, intensive development of molecular-cytogenetic methods gives us hope that more species examined will allow further reconsideration of the pathways of Orthoptera chromosome evolution, which led to the formation of similar karyotype structure of Pyrgomorphidae and Pamphagidae grasshoppers.

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SHORT COMMUNICATION

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Heterochromatin variation and LINE-1 distribution in *Artibeus* (Chiroptera, Phyllostomidae) from Central Amazon, Brazil

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Abstract

Species in the subgenus *Artibeus* Leach, 1821 are widely distributed in Brazil. Conserved karyotypes characterize the group with identical diploid number and chromosome morphology. Recent studies suggested that the heterochromatin distribution and accumulation patterns can vary among species. In order to assess whether variation can also occur within species, we have analyzed the chromosomal distribution of constitutive heterochromatin in *A. planirostris* (Spix, 1823) and *A. lituratus* (Olfers, 1818) from Central Amazon (North Brazil) and contrasted our findings with those reported for other localities in Brazil. In addition, Ag-NOR staining and FISH with 18S rDNA, telomeric, and LINE-1 probes were performed to assess the potential role that these different repetitive markers had in shaping the current architecture of heterochromatic regions. Both species presented interindividual variation of constitutive heterochromatin. In addition, in *A. planirostris* the centromeres of most chromosomes are enriched with LINE-1, colocated with pericentromeric heterochromatin blocks. Overall, our data indicate that amplification and differential distribution of the investigated repetitive DNAs might have played a significant role in shaping the chromosome architecture of the subgenus *Artibeus*.

Keywords
Bats, chromosomes, cytogenetics, FISH, repetitive DNA, Stenodermatinae

Introduction

Currently, three species of large body size *Artibeus* (subgen. *Artibeus* Leach, 1821) are found in the Brazilian Amazon region: *A. obscurus* (Schinz, 1821), *A. lituratus* (Olfers, 1818), and *A. planirostris* (Spix, 1823) (Marques-Aguiar 2007, Gardner 2008). These bat species occur in sympatry in most Brazilian environments, and display considerable morphological variation along their geographic distribution. The overlapping measurements of external morphological characters can still lead to misidentification between *A. planirostris* and *A. obscurus* in the field. On the other hand, *A. lituratus* and *A. planirostris* are easily distinguishable morphologically (Gardner 2008). Nevertheless, cranial features and a more detailed examination of voucher specimens usually provide diagnostic characters for species identification (Ortega and Castro-Arellano 2001, Haynes and Lee 2004, Lim et al. 2004).

Cytogenetic studies in all species of the subgenus *Artibeus* revealed a conserved karyotype, with diploid number (2n) of 30 chromosomes for females and 31 for males, with fundamental number, FNa = 56 (Baker 1967, Souza and Araújo 1990, Noronha et al. 2001, Santos et al. 2002, Baker et al. 2003, Calixto et al. 2014). The 2n difference between females and males is due to a XX/XY_1Y_2 multiple sex chromosome system shared by most species of the subfamily Stenodermatinae P. Gervais, 1856 (Tucker and Bickham 1986, Noronha et al. 2001, Rodrigues et al. 2003, Pieczarka et al. 2013). Although the overall patterns of classical cytogenetic markers (including G- and C- banding, and Ag-NOR staining) are fairly well investigated, variation of constitutive heterochromatin (CH) distribution was just recently reported among *Artibeus* species (Lemos-Pinto et al. 2012). In their work, Lemos-Pinto et al. (2012) investigated the CH distribution in the karyotypes of *Artibeus* from the state of Pernambuco (Northeast Brazil), and proposed that the CH patterns were species-specific. However, although independent studies focused on species cytogenetic characterizations at the local level, no study has targeted the detection of interindividual CH variation in *Artibeus* within and among different Brazilian regions.

Chromosomal evolution, including variation in the patterns of CH distribution is usually associated with distinct repetitive DNA dynamics. Therefore, *in situ* mapping of repetitive markers (e.g., 18S rDNA and telomeric sequences and interspersed repetitive elements) can significantly contribute to the understanding of the evolution of genome architecture, as well as to the identification of intraspecific polymorphism in karyotypes otherwise conserved (Baker and Bickham 1980, Morielle and Garcia 1988, Varella-Garcia et al. 1989, Souza and Araújo 1990, Baker et al. 2003, Lemos-Pinto et al. 2012). Howerver, there is still a lack of studies correlating the localization of CH and repetitive elements in bats. This is particularly true for transposable elements (TEs), despite their significant incidence in vertebrate genomes, and their potential to drive heterochroma-

tin formation (Gentles et al. 2007, Chalopin et al. 2015, Sotero-Caio et al. 2017). For example, although LINE (Long Interspersed Nuclear Element) retrotransposons are the most prevalent TEs in mammals, their chromosomal distribution were described for only four bat species (Parish et al. 2002, Sotero-Caio et al. 2015).

In the present study, we investigate whether there is CH variation within Central Amazon populations (North Brazil) of two *Artibeus* species (*A. planirostris* and *A. lituratus*), as well as CH variation among representatives from Amazonian and other Brazilian regions. Furthermore, we have mapped rDNA and telomeric sequences on the karyotypes of both species to assess whether these sequences contribute to the architecture of centromeres and other positive heterochromatin blocks. As our final goal, we investigated the chromosomal distribution of LINE-1 sequences in *A. planirostris* chromosomes to i) compare with patterns described for other phyllostomid species, and ii) correlate the distribution of these sequences with the CH pattern observed for individuals in the same population.

Materials and methods

The specimens used in this investigation were collected during expeditions conducted in 2009. The sampling locations were not within protected areas, and *Artibeus* species used in this study are not listed as endangered at national or local levels. Our sampling included specimens of *A. planirostris* collected in an urban fragment at the National Institute of Amazonian Research (INPA) (03°05'51.1"S, 59°59'8.4"W), and at "Bons Amigos" Farm (Km 14 of BR 174; 02°50'37"S, 60°03'58"W). Furthermore, we collected individuals of *A. lituratus* at "Bons Amigos" Farm, Amazonas State, Brazil (Table 1). Voucher specimens and cytological material were deposited at the "Laboratório de Genética Animal" at INPA.

Mitotic chromosomes were obtained from bone marrow cells using the *in vivo* method (Lee and Elder 1980, Varella-Garcia and Taddei 1989). C-banding patterns and nucleolus organizing region (NOR) locations were determined according to Sumner (1972), and Howell and Black (1980), respectively. The FISH probes were prepared by PCR using primers to amplify the 18S ribosomal gene (18SF, 5' CCGCTTTGGTGCTCTTGAT 3'; 18SR, 5' CCGAGGACCTCATAAACCA 3') (Gross et al. 2010), the telomeric sequences $(TTAGGG)_n$ (Ijdo et al. 1991), and LINE-1 (L1R, 5' ATTCTRTTCCATTGGTCTA 3'; L1F, 5' CCATGCTCATSGATTGG 3') (Waters et al. 2004) (Table 1). The PCR products were labeled by nick translation using biotin kit (Bio-Nick ROCHE). FISH procedures followed Pinkel et al. (1986) with modifications: mitotic chromosomes were denatured in 70 % formamide/0.6X SSC (pH 7.0) for 5 minutes at 70 °C; the hybridization mix applied per slide contained 200 ng of probe, 10 % dextran sulfate, 2 X SSC and 50 % formamide in a final volume of 40 µl. Slides were incubated overnight at 37 °C. Post-hybridization washes were carried out at 42 °C in 15% formamide/0.2X SSC for five minutes. Detection was performed with avidin-FITC (fluorescein isothiocyanate) conjugate (Sigma, St Louis, MO, USA), followed by counterstaining with Propidium Iodide (0.2%) and mounting in Vectashield (Vector, Burlingame, CA, USA).

Table 1. List of specimens and respective methodologies applied in the present study. Sampling localities for each voucher are given in the last column.

Species	Voucher ID	Sex	Giemsa Staining	C-banding	Ag-NOR Staining	18S FISH	Telomeric FISH	LINE-1 FISH	Sampling Site
A. planirostris	EMS05	♂	X	X	–	–	–	–	Urban fragment at INPA
	EMS06	♂	X	X	X	–	–	–	Urban fragment at INPA
	EMS07	♂	X	X	X	X	X	–	Urban fragment at INPA
	EMS09	♀	X	X	X	X	X	X	Urban fragment at INPA
	EMS10	♂	X	X	–	–	–	X	Urban fragment at INPA
	EMS18	♀	X	X	X	X	X	–	"Bons Amigos" Farm
	EMS14	♂	X	X	X	X	X	–	"Bons Amigos" Farm
	EMS17	♂	X	X	X	–	–	–	"Bons Amigos" Farm
A. lituratus	EMS15	♀	X	X	X	X	X	–	"Bons Amigos" Farm
	EMS16	♂	X	X	X	X	X	–	"Bons Amigos" Farm
	EMS19	♂	X	X	X	X	–	–	"Bons Amigos" Farm

The chromosomes were analyzed using an Olympus BX51 microscope, and the metaphases were captured with an Olympus DP70 digital camera using IMAGE-PRO MC 6.0 software. The images were processed using ADOBE PHOTOSHOP CS3 program, and the chromosomes were measured using the IMAGE J (Schneider et al. 2012). The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a), in descending size order (Levan et al. 1964). The fundamental number was based on the number of autosomal arms (FNa), as described by Gardner and Patton (1976).

C-banding reports from Souza and Araújo (1990), Rodrigues et al. (2003), and Lemos-Pinto et al. (2012) were assessed to detect inter- and intraspecific CH variation among specimens from different Brazil regions.

Results and discussion

Classical Cytogenetics and Constitutive Heterochromatin Variation

Classical Giemsa staining did not uncover structural variation between the karyotypes of *A. planirostris* and *A. lituratus* from Amazonas. Both species have the same diploid

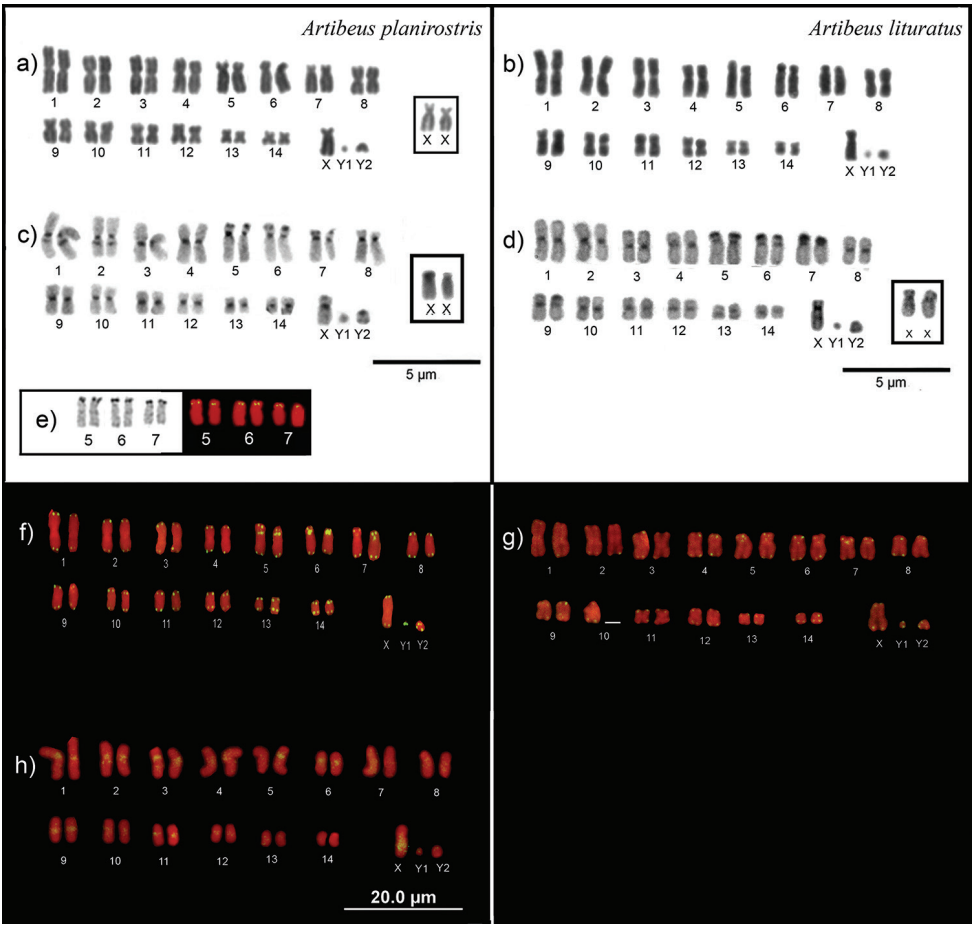

Figure 1. Karyotypes of *A. planirostris* (**a, c, e, f, h**) and *A. lituratus* (**b, d, g**). Conventional staining (**a, b**); C-banding patterns (**c, d**); Ag-NOR staining (left) and FISH with rDNA 18S (rigth; **e**), FISH using telomeric repeats as probes (**f, g**), FISH with probes from the open reading frame (ORF) II of LINE-1 from *A. planirostris* (**h**).

(2n = 30/31, XY_1Y_2) and fundamental numbers (FNa = 56), with 11 metacentric and three subtelocentric chromosome pairs (22m+6st+XX/XY_1Y_2). The X chromosome was a medium submetacentric, Y_1 had a dot-like morphology, and Y_2 was a small acrocentric (Fig. 1a, b).

Despite having the same karyotype, slight differences of constitutive heterochromatin distribution were observed, especially for sex chromosomes, between *A. planirostris* and *A. lituratus*. C-banding revealed CH in the centromeric region of all autosomes of both species. Additionally, in *A. planirostris* small heterochromatic blocks were observed in the proximal region of long arms on two metacentric chromosomes (1st and 2nd pairs), as well as in the distal region of short arms on three subtelocentric pairs (5th, 6 th and 7 th), which are adjacent to the location of active Ag-NORs. The Y_1 chromosome

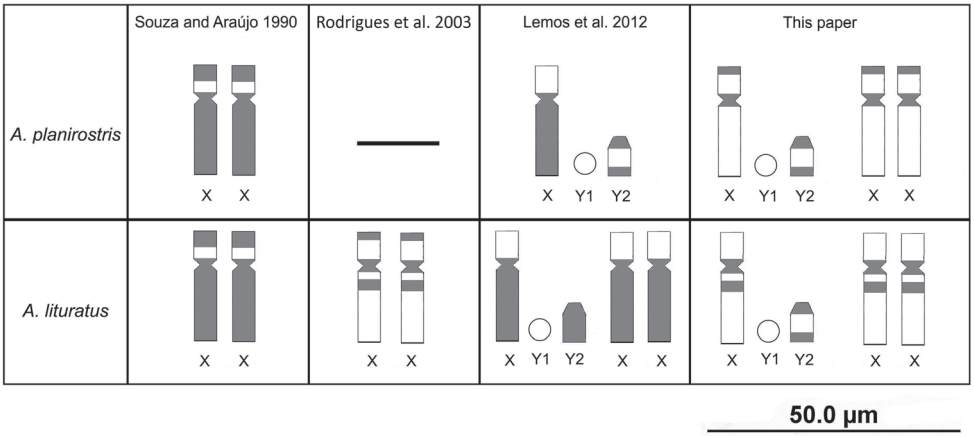

Figure 2. Schematic representation of *A. planirostris* and *A. lituratus* sex chromosomes showing C-banding variation reported in different studies. Gray shading corresponds to heterochromatin and the euchromatic regions are depicted in white color.

was euchromatic, and the Y_2 had centromeric heterochromatin and additional blocks on the long arms. Likewise, the X chromosome showed centromeric heterochromatin and blocks on the short arms. The long arm of the X chromosome however, was not particularly enriched with heterochromatin (Fig. 1c). A similar pattern of heterochromatin distribution on the autosomes was observed for *A. lituratus,* with large heterochromatic blocks on short arms of chromosome pairs 5th–7th (Fig. 1d). However, the 1st and 2nd pairs showed only centromeric blocks. The X chromosome showed centromeric heterochromatin, as well as CH blocks on the long arms. Finally, the patterns of CH distribution on Y_1 and Y_2 chromosomes were similar to those of *A. planirostri*s.

C-banding did not disclose within-species variation in our Amazonian samples. The observed CH patterns are, however, distinct from those reported in non-Amazonian indivuduals, indicating the existence of interindividual variation in both *Artibeus* species (Souza and Araujo 1990, Rodrigues et al. 2003, Lemos-Pinto et al. 2012). For example, we did not detect heterochromatic blocks on the distal region of the 9th pair in individuals of either species as previously described by Lemos-Pinto et al. (2012) for samples collected in Pernambuco state. Furthermore, our results indicate that heterochromatin distribution on X chromosomes can vary not only among species, but also within species (Fig. 2). In this regard, two patterns were previously reported for specimens of *A. planirostris* from Pernambuco, Northeastern Brazil: (i) heterochromatic sites at centromeres, long arm, and distally on the short arm (Souza and Araújo 1990); and (ii) heterochromatic sites at the centromere and long arm (Lemos-Pinto et al. 2012). Both results differ from the data presented here because the long arm of X chromosomes of Amazonian specimens lacked evident CH blocks. Similarly, specimens of *A. lituratus* from Pernambuco have two patterns: (i) centromeric, plus distal on the short arm, and long arm (Souza and Araújo 1990); and (ii) centromere and long arm (Lemos-Pinto et al. 2012). Additionally, in specimens collected in Pará state

(Northern Brazil), the X heterochromatin was centromeric, distal on the short arm, and interstitial on the long arm (Rodrigues et al. 2003).

Although the number of analyzed individuals (eight *A. planirostris* and three *A. lituratus*) from the Amazon is too low to make generalizations, the similar number and location of heterochromatic blocks between individuals from Pará and Amazonas (both Northern Brazil), might indicate that specimens from the same ecogeographic regions have similar CH patterns. Pará and Amazonas states are contiguous and covered mostly by Amazon rainforest, whereas Pernambuco is a coastal state, separated from the Amazon by dry forests, and transitional environments, which might serve as mild dispersion barriers. Therefore, additional studies including large sampling are required to test the hypothesis that CH variation occurs by differential turnover of repetitive DNA (derived either by their removal/amplification or by recombination), reinforced by geographical barriers through the distributional gradient of species.

Ag-NOR Staining and In Situ Hybridization with Repetitive Probes

Silver nitrate staining and 18S rDNA FISH detected NORs at multiple sites on chromosomes of both species, more specifically distally on the short arms of the 5th, 6th and 7th pairs (Fig. 1e; data not shown for *A. lituratus*). The present Ag-NORs and 18S rDNA results agree with previously reported data (Morielle and Varella-Garcia 1988, Souza and Araújo 1990, Santos et al. 2002, Lemos-Pinto et al. 2012, Calixto et al. 2014). Many species in the subfamily Stenodermatinae have multiple NORs, which is considered a derivative condition. For example, this condition regards other *Artibeus* species (subtribe Artibeina H. Allen, 1898), *Uroderma bilobatum* W. Peters, 1866, *U. magnirostrum* Davis, 1968, *Vampyriscus bidens* (Dobson, 1878), and *Vampyressa thyone* O. Thomas, 1909 (subtribe Vampyressina Baker et al., 2016), as well as *Centurio senex* Gray, 1842 (subtribe Stenodermatina Gervais, 1856) (Baker et al. 1992, Santos et al. 2002, Gomes et al. 2016). However, the multiple NORs of the above mentioned groups are not necessarily located on homologous chromosomes (orthologous chromosome regions). Additionally, basal clades within Stenodermatinae (e.g., genus *Sturnira* Gray, 1842), and species in the same tribe as *Artibeus* (e.g., *Platyrrhinus* Saussure, 1860 and *Mesophylla* O. Thomas, 1901 species) do not have multiple NORs (Gomes et al. 2016). Therefore, we hypothesize that the presence of NORs on the three particular chromosome pairs of the analyzed species was a feature of the common ancestor of all *Artibeus* (Santos et al. 2002, Baker et al. 2016, Gomes et al. 2016). As another mammals, *Artibeus* NORs collocate (are adjacent) with heterochromatin and are likely associated with the amplification of heterochromatin in non centromeric regions.

In both species, *in situ* hybridizations detected $(TTAGGG)_n$ telomeric sequences in all telomeres. Additionally, both species shared centromeric signals on three subtelocentric pairs (pairs 5th, 6th and 7th; Fig. 1f, g). There are two potential explanations for the presence of telomeric sequences in interstitial position (ITS): (i) these sequences might be telomere motifs reallocated from the terminal region of a chromosome to

another chromosome or chromosome position; and (ii) the ITS presence on the centromere derives from reorganization of repetitive sequences (satellite DNA) composing these regions, which could also indicate the presence of centromeric hotspots of recombination during *Artibeus* karyotype evolution (Nanda and Schmid 1994, Multani et al. 2001, Metcalfe et al. 2007, Faria et al. 2009, Kasahara 2009, Silva et al. 2016, Teixeira et al. 2016). The karyotypic evolution of *Artibeus* is considered extremely conservative, however the formation of the ancestral karyotype of the subfamily Stenodermatinae required extreme reshuffling (Baker and Bickham 1980, Pieczarka et al. 2013). Therefore, the ITS allocated on the pairs 5 th–7 th for both species might be remnants of chromosome rearrangements that have been amplified or lost differentially in different Stenodermatinae species. Calixto et al. (2014) have shown that many phyllostomids species present ITS, regardless of their trend of karyotypic evolution. For example, species with a conservative karyotypic evolution, such as *Trachops cirrhosus* (Spix, 1823) and *Phyllostomus elongatus* (É. Geoffroy St. -Hilaire, 1810) (both in the subfamily Phyllostominae Gray, 1825) present ITS, which suggest lineage-specific events of amplification of these sequences can occur independently. Furthermore, it is noteworthy that pairs 5th–7th have ITS, cetromeric and non-centromeric CH blocks, as well as the NORs in all *Artibeus* specimens analyzed, suggesting that differential dynamics of heterochromatin DNA in these particular chromosomes might have played a role in the establishment of their shared distinct architecture, when compared to other autosomes. Refined investigation of these chromosomes at the sequence level will help disclosing whether differential heterochromatin composition contributed to the establishment of centromeric ITS.

LINE-1 mapping on *A. planirostris* chromosomes revealed FISH signals near the centromere of most autosomes, except pairs 4, 7, 8, 13 and 14. (Fig. 1g). The centromeric FISH results were consistent in all analyzed individuals (n=4) and the centromeric pattern contrasts with the longitudinal distribution previously shown for most mammals, including other phyllostomid bats, *Carollia brevicauda* (Schinz, 1821), *Lophostoma occidentalis* (Davis & Carter, 1978), and *Gardnerycteris crenulatum* (É. Geoffroy St. -Hilaire, 1803) (Parish et al. 2002, Dobigny et al. 2006, Ferreri et al. 2011, Pieczarka et al. 2013, Sotero-Caio et al. 2015). Centromeric accumulation of retroelements in mammalian chromosomes is rare, but some cases have been described. For instance, Waters et al. (2004) found LINE-1-positive centromeres in the karyotypes of African mammals. Likewise, Sotero-Caio et al. (2015) showed centromeric LINE-1 accumulation in chromosomes of the phyllostomid bat *Tonatia saurophila* Koopman & Williams, 1951 (Phyllostominae). It was hypothesized that this unusual distribution might have contributed to the high degree of chromosomal reorganization in the genus *Tonatia* Gray, 1827. From our data, it is still premature to state that this retroelement or sequences derived from it are constitutional components of core centromeres. Similarly, because our probes comprised only a partial LINE-1 sequence, we cannot conclude that functional elements are contributing to the centromere dynamics of *Artibeus*. Despite the uncertainty on what factors were responsible for the massive "colonization" of LINE-1s at centromeres, processes such as gene conversion, which

promote homogenization of centromeric sequences are expected to facilitate the maintenance of LINE-1 sequences in high copy numbers in this region (Shi et al. 2010).

We noticed inconsistent patterns when comparing the co-distribution of heterochromatin blocks and LINE-1 elements. Namely, in all analyzed individuals, interstitial CH blocks have LINE-1 signals in the second chromosome pair but not pair 1. Thus, non-centromeric heterochromatin formation on chromosomal arms of *A. planirostris* could be a result of amplification of different types of repeats (e.g. LINEs vs. satellite DNA) in specific chromosomes (Parish et al. 2002, Dobigny et al. 2006, Sumner 2008, Shi et al. 2010, Ferreri et al. 2011, Carbone et al. 2012).

The Y_1 and Y_2 sex chromosomes presented weak FISH signals, contrasting with the strong signal throughout the long arm of the X (Fig. 1h). LINE-1 accumulation on X chromosomes is a pattern observed in all mammal species, including other phyllostomid bats (Lyon 1998, Parish et al. 2002, Dobigny et al. 2006, Cantrell et al. 2008, Liu et al. 2011, Sotero-Caio et al. 2015). Parish et al. (2002) investigated the concentration of LINE-1 in *C. brevicauda* chromosomes, which also presents a multiple sex chromosome system. They found that the original X chromosome had higher levels of LINE-1 accumulation than the translocated autosome. The X-autosome translocation of *Artibeus* is different from that observed in *C. brevicauda*, with the small autosome component representing the short arm of *A. planirostris* X chromosome. In agreement with Parish et al. (2002) findings and Lyon hypothesis, we identified that Xq of *A. planirostris* had a significant accumulation of LINE-1, corresponding exactly to the original X chromosome. The LINE-1 accumulation on Y-chromosomes seems to be restricted to centromeric regions. In this case, we expect that similarly to other mammals, the original Y is mostly constituted of repeats other than retroelements, and that Y_2 pattern corresponds to that observed for other autosomes due to its autosomal origin.

Transposable element activity and accumulation have been linked to chromosomal rearrangements and can be directly or indirectly associated with speciation events (Lim and Simmons 1994, Dörner and Pääbo 1995, Gray 2000, Dobigny et al. 2004, Waters et al. 2004, Carbone et al. 2012, 2014). In addition, the dispersal dynamics of TEs are related to biological functions such as gene regulation, chromosomal rearrangements, X inactivation on females and horizontal transfer events among closely or distantly related species (Lyon 1998, Ostertag and Kazazian Jr 2001, Chow et al. 2010) . The distribution of repetitive elements in *Artibeus* might have played a significant role in shaping the chromosome architecture of the genus, and we are still unsure if this trend at centromeres can be observed in other species of Stenodermatinae. Because the LINE-1 accumulation patterns differ in the bat species analyzed to date (Parish et al. 2002, Sotero-Caio et al. 2015, present study), we hypothesize that these elements constitute potential contributors to the great karyotype reshuffling presented by some phyllostomid taxa since the divergence of their ancestral karyotype. Overall, our data suggest that different mechanisms might have contributed to the karyotype evolution of phyllostomid bats, explaining why only *Artibeus* and *Tonatia* species, but not *C. brevicauda*, *L. occidentalis*, and *G. crenulatum* differred in the patterns of LINE-1 distribution.

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Cytogenetic analysis of five Ctenidae species (Araneae): detection of heterochromatin and 18S rDNA sites

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Abstract

The present study aimed to cytogenetically analyse five Ctenidae species *Ctenus ornatus* (Keyserling, 1877), *Ctenus medius* (Keyserling, 1891), *Phoneutria nigriventer* (Keyserling, 1891), *Viracucha andicola* (Simon, 1906), and *Enoploctenus cyclothorax* (Philip Bertkau, 1880), from Brazil. All species presented a 2n♂ = 28 except for *V. andicola*, which showed 2n♂ = 29. Analysis of segregation and behavior of sex chromosomes during male meiosis showed a sex chromosome system of the type X_1X_20 in species with 28 chromosomes and $X_1X_2X_30$ in *V. andicola*. C banding stained with fluorochromes CMA_3 and DAPI revealed two distributions patterns of GC-rich heterochromatin: (i) in terminal regions of most chromosomes, as presented in *C. medius*, *P. nigriventer*, *E. cyclothorax* and *V. andicola* and (ii) in interstitial regions of most chromosomes, in addition to terminal regions, as observed for *C. ornatus*. The population of Ubatuba (São Paulo State) of this same species displayed an additional accumulation of GC-rich heterochromatin in one bivalent. Fluorescent in situ hybridization revealed that this bivalent corresponded to the NOR-bearing chromosome pair. All analyzed species have one bivalent with 18S rDNA site, except *P. nigriventer*, which has three bivalents with 18S rDNA site. Karyotypes of two species, *C. medius* and *E. cyclothorax*, are described for the first time. The latter species is the first karyotyped representative of the subfamily Acantheinae. Finally, 18S rDNA probe is used for the first time in Ctenidae at the present study.

Keywords

C-banding, FISH, fluorochrome, meiosis, spider cytogenetics, sex chromosomes

Introduction

Ctenidae is a family of Araneae distributed throughout the tropical region of the planet (World Spider Catalog 2017). This family includes wandering and nocturnal spiders, with some species of medical interest, such as those of the genus *Phoneutria* Perty, 1833 (Ministério da Saúde 2017). Ctenidae is divided into five subfamilies, namely Acanthocteninae, Viridasiinae, Cteninae, Calocteninae, and Acantheinae (Silva-Dávila 2003; Polotow and Brescovit 2014). Although ctenids are of great ecological and medical importance, studies on their cytogenetics are scarce (Table 1) and cytogenetic data for the last two subfamilies are not available to date.

Three karyotypes have been observed in the family: (i) 2n♂ = 22 (20 + X_1X_20); (ii) 2n♂ = 28 (26 + X_1X_20); and (iii) 2n♂ = 29 (26 + $X_1X_2X_30$) (Table 1). The sex chromosome systems (SCS) in spiders are considered highly diverse by many authors (Král et al. 2006; 2011; Araujo et al. 2012) ranging from simple systems, such as XY or X0, to multiple SCS as X_nY_n or X_n0 (Araujo et al. 2017). Based on findings in a specimen of *Ctenus ornatus* (Keyserling, 1877) Araujo et al. (2014) suggested that the $X_1X_2X_30$ system in Ctenidae, might have arisen from a supernumerary chromosome and, according to literature evidence, this system arose repeatedly in the evolutionary history of Entelegynae and its conversion into the X_1X_20 system and vice-versa is a recurring event. Bole-Gowda (1952) also suggested the involvement of a supernumerary element in the origin of the X_3 chromosome in Sparassidae species. Other hypotheses on the conversion of a X_1X_20 into a $X_1X_2X_30$ were also proposed by some authors (Pätau 1948; Postiglioni and Brum-Zorrilla 1981; Parida and Sharma 1986). The conversion of a $X_1X_2X_30$ into a X_1X_20 was firstly proposed in the spider genus *Malthonica* Simon, 1898 (Agelenidae) by Král (2007), suggesting that tandem fusions occurred in this process.

Chromosome banding techniques, as identification of nucleolus organizer regions (NORs) using silver nitrate impregnation, have been performed in Ctenidae. Araujo et al. (2014) found a single terminal NOR on one autosomal pair in *C. ornatus* and *Phoneutria nigriventer* (Keyserling, 1891), and on two pairs in *Viracucha andicola* (Simon, 1906). Kumar et al. (2016) also detected NORs on two autosomal pairs in *Ctenus indicus* (Gravely, 1931). However, molecular cytogenetic studies are scarce in spiders. There have been only five studies about distribution of some sequences using fluorescence *in situ* hybridization (FISH): location of 18S rDNA sites in *Wadicosa fidelis* (O. Pickard-Cambridge, 1872) (Lycosidae) (Forman et al. 2013) and *Brachypelma albopilosum* Valerio, 1980 (Theraphosidae) (Král et al. 2013); 5S rDNA sites in *Oxyopes sertatus* L. Koch, 1878 (Oxyopidae) (Suzuki and Kubota 2011); mapping of silk genes in *Latrodectus hesperus* Chamberlin & Ivie, 1935 and *Latrodectus geometricus* C. L. Koch, 1841 (Theridiidae) (Zhao et al. 2010); and ocurrence of telomeric repeats in *Brachypelma albopilosa* Valerio, 1980 (Vítková et al. 2005).

Considering the great importance of ctenids and the scarcity of cytogenetic studies in the group, our study analyzed the mitotic and meiotic chromosomes of five species

Table 1. Cytogenetic data of Ctenidae species, updated from Araujo et al. (2014), including the data of present study. NOR = nucleolus organizer region.

Species	Karyotype (♂)	NORs		Reference
		Silver Nitrate	detection of 18S rDNA	
Acantheinae				
Enoploctenus cyclothorax (Bertkau, 1880)	28, X_1X_20		2	Present study
Acanthocteninae				
Nothroctenus sp.	29, $X_1X_2X_30$			Araujo et al. 2014
Viracucha andicola (Simon, 1906)	29, $X_1X_2X_30$	4		Araujo et al. 2014
			2	Present study
Cteninae				
Anahita fauna Karsch, 1879	29, $X_1X_2X_30$			Chen, 1999
Ctenus indicus (Gravely, 1931)	28, X_1X_20	4		Kumar et al. 2016
Ctenus medius Keyserling, 1891	28, X_1X_20		2	Present study
Ctenus ornatus (Keyserling, 1877)	28, X_1X_20	2		Araujo et al. 2014
Ctenus sp.	28, X_1X_20			Araujo et al. 2014
Parabatina brevipes (Keyserling, 1891)	28, X_1X_20			Araujo et al. 2014
Phoneutria nigriventer (Keyserling, 1891)	28, X_1X_20	2		Araujo et al. 2014
			6	Present study
Viridasiinae				
Asthenoctenus borelli Simon, 1897	22, X_1X_20			Araujo et al. 2014

of this family. To understand better the karyotype structure in this group of spiders, we evaluated the behavior of sex chromosomes, heterochromatin composition/distribution pattern, and the location of 18S rDNA sites.

Material and methods

Specimen deposition

Adults and juveniles of five ctenid species from different collection sites in Brazil were analyzed, as listed in Table 2. Specimens were deposited in the arachnological collection of the Laboratório Especial de Coleções Biológicas at Instituto Butantan (IBSP, curator A. D. Brescovit), São Paulo/SP (São Paulo state), Brazil.

Chromosome preparations and banding

Chromosomal preparations were obtained according to Araujo et al. (2008), with some modifications as follows. After the fixation, testes were dissociated in a drop of 60% acetic acid on the surface of a microscope slide and covered with a coverslip, pressed and immersed in liquid nitrogen to allow the removal of the coverslip. The diploid number was determined by counting 30 meiotic and mitotic cells. The morphology of

Table 2. List of collected species, with the number of the individuals, collection sites, and voucher numbers. PR = Paraná State. SP = São Paulo State.

Species	Individuals (♂)	Collection Site	Voucher Number
Ctenus medius	5	Londrina (23°19'37.5"S, 51°12'13.4"W), PR	166439, 167462, 167463, 167466, 167490
Ctenus ornatus	11	Londrina (23°19'37.5"S, 51°12'13.4"W), PR	166426–166430, 166440–166442, 166449, 166458–166459
	9	Céu Azul (25°09'15.8"S, 53°50'42.1"W), PR	166399–166401, 167467–167470, 167476–167477
	2	Foz do Iguaçu (25°37'41.2"S 54°27'47.2"W), PR	166416, 167465
	4	Ubatuba (23°24'14.3"S 45°03'54.0"W), SP	166453-166454, 167402, 167406
Enoploctenus cyclothorax	3	Céu Azul (25°09'15.8"S, 53°50'42.1"W), PR	166397, 166398, 166407
Phoneutria nigriventer	5	Londrina (23°19'37.5"S, 51°12'13.4"W), PR	166441, 167407, 167489, 167494, 167495
	1	Céu Azul (25°09'15.8"S, 53°50'42.1"W), PR	166412
	1	Foz do Iguaçu (25°37'41.2"S 54°27'47.2"W), PR	167405
Viracucha andicola	6	Londrina (23°19'37.5"S, 51°12'13.4"W), PR	166434, 166445, 166447, 167398–167400
	2	Céu Azul (25°09'15.8"S, 53°50'42.1"W), PR	166411, 166413

chromosomes was classified according to Levan et al. (1964), using the MicroMeasure version 3.3 software (Reeves and Tear 2000). To determine the heterochromatin location and its composition, the slides were submitted to C-banding following Sumner (1972) and subsequently stained with base-specific fluorochromes, chromomycin A_3 (CMA_3) and 4', 6-diamidino2-phenilindole (DAPI), according to the procedure described by Schweizer (1980).

18S rDNA probe generation

Genomic DNA of *C. ornatus* was extracted using a standard phenol/chloroform procedure (Sambrook and Russell 2006). A polymerase chain reaction (PCR) was performed with the primers of 18S rDNA, forward: CGAGCGCTTTTATTAGACCA and reverse: GGTTCACCTACGGAAACCTT, as described by Forman et al. (2013). Another pair of primers was designed in the Primer3Plus software (Untergasser et al. 2007) to allow the complete amplification of the 18S rDNA fragment, forward: TCTGTCTCGTGCGGCTAAAC and reverse: GATCCATTGGAGGGCAAGTC. The PCR reaction contained diluted genomic DNA, *Taq* buffer, 0.8 mM dNTP mix, 4 mM $MgCl_2$, 5 pmol of each primer, and 2.5 U of *Taq* polymerase (Invitrogen) for a reaction of 25 µl. The amplification was performed with an initial denaturation of 2 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 60 °C, and 5 min at 72 °C until completion. The 18S rDNA was purified by agarose gel using the Pure

Link-Quick Gel Extraction Kit (Invitrogen). The DNA fragment generated by the pair of primers described by Forman et al. (2013) was cloned using the kit pGEM-T Easy Vector System (Promega) in a suitable strain of *Escherichia coli* (TOP 10) and the insert was sequenced by the ABI-Prism 3500 Genetic Analyzer (Applied Biosystems).

The sequence was analyzed using the free software BioEdit, version 7.2.5 (Hall 2013). The rDNA sequence of 1280 pb, obtained from *C. ornatus*, was submitted to BLASTN (Altschul et al., 1990) in the National Center for Biotechnology Information (NCBI) database, through web site (http://www.ncbi.nlm.nih.gov/blast), to verify the homology with sequences of 18S rDNA from spiders and demonstrated 99% of homology with *Phoneutria fera* Perty, 1833 (accession KY016373.1) in the GenBank. The sequence was deposited on NCBI, accession KT698160.1.

Fluorescence *in situ* hybridization

The 18S rDNA sites were identified using the FISH technique according to Pinkel et al. (1986) and Gouveia et al. (2013), with the following modifications. After dehydration, the slides were treated with formamide 15%/SSC for 10 min and subsequently in pepsin (0.005 mg/mL) for 20 min. Probes were labeled with the Dig-Nick Translation kit (Invitrogen) and detected by the monoclonal anti-digoxigenin antibody conjugated to rhodamine (Roche Applied Science, Indianapolis, IN). Preparations were counterstained with DAPI. In the Ubatuba *C. ornatus* population, the slides were stained after a FISH procedure with CMA_3 and DAPI to visualize the association between 18S rDNA sites and GC-rich blocks. Finally, the slides were analyzed in an epifluorescence microscope (Leica DM 2000), equipped with a digital camera Moticam Pro 282B. The images were captured using the Motic Images Advanced software, version 3.2.

Results

Ctenus ornatus, *Ctenus medius* Keyserling, 1891, *Phoneutria nigriventer,* and *Enoploctenus cyclothorax* (Bertkau, 1880) exhibited 2n♂ = 28, as observed in mitotic metaphases (Fig. 1A, E, I, M), whereas *Viracucha andicola* presented 2n♂ = 29 (Fig. 1Q). All chromosomes were identified in metaphases II as acrocentric (Fig. 1D, H, L), except for *E. cyclothorax* and *V. andicola*, in which it was difficult to determine accurately the morphology of all chromosomes (Fig. 1P, T).

At male diakinesis 13 bivalents in all species were found and two univalent X in parallel association in the species with 28 chromosomes (Fig. 1C, G, K, O) and three univalent X in the species with 29 chromosomes (Fig. 1S). Three sex chromosomes in *V. andicola* showed parallel association (Fig. 1S-box). In some plates at pachytene and diplotene X are not associated in species with the two X chromosomes (Fig. 1C, G, K, O-boxes). Species with 2n♂ = 28 showed metaphases II with 13 and 15 chromosomes (Fig. 1D, H, L, P), and species with 2n♂ = 29 showed cells with 13 and 16

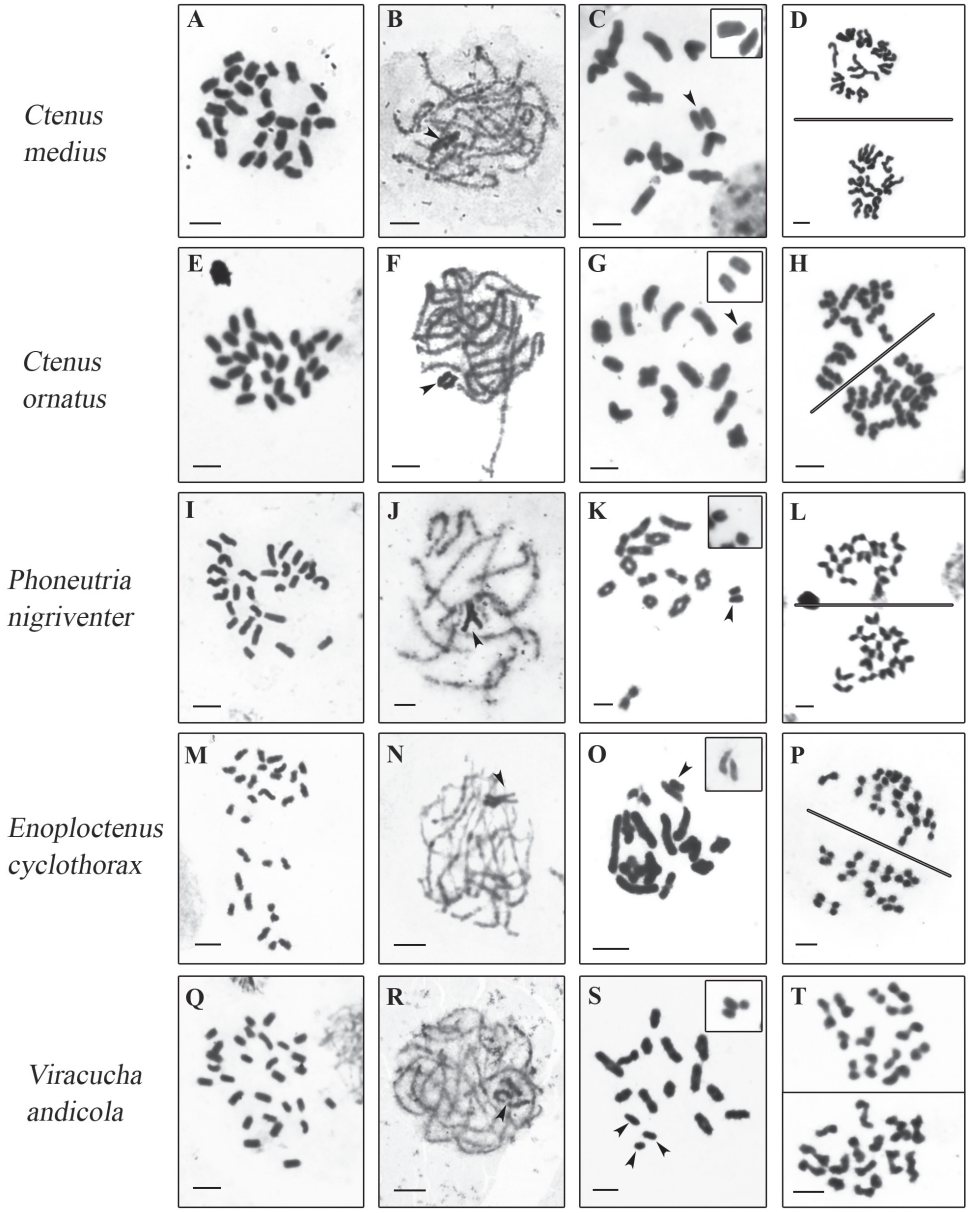

Figure 1. Male mitotic and meiotic cells of Ctenidae species stained with Giemsa. Boxes – X chromosomes without association (**C, G, K, O**), and with association (**S**). *C. medius* (**A–D**), *C. ornatus* (**E–H**), *P. nigriventer* (**I–L**), *E. cyclothorax* (**M–P**), *V. andicola* (**Q–T**). The arrowheads show sex chromosomes. Mitotic metaphases with 2n = 28 (**A, E, I, M**) and 2n =29 (**Q**). Pachytene cells (**B, F, J, N, R**) with positively heteropycnotic sex chromosomes. Diakinesis cells (**C, G, K, O, S**), note parallel association of two X chromosomes (**C**, **G**, **K**, **O**) or three X chromosomes without association (**S**). Metaphase II cells with n = 13 and n = 13 + X_1X_2 **(D, H, L, P)** and n = 13 and n = 13 + $X_1X_2X_3$ **(T)**. Bar = 10 μm.

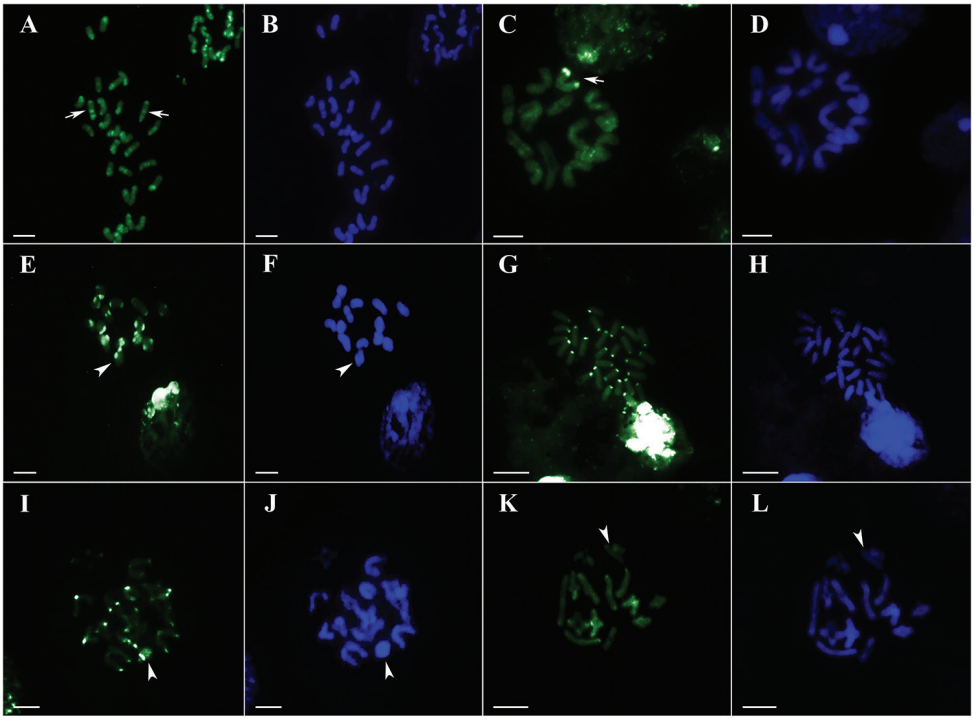

Figure 2. Ctenidae male mitotic and meiotic cells, C-banding and staining with base-specific fluorochromes CMA_3 (**A, C, E, G, I, K**) and DAPI (**B, D, F, H, J, L**). Arrowhead - X chromosomes. **A, B** mitotic metaphase of *Ctenus ornatus*, 28 chromosomes, arrow – interstitial CMA_3^+ region **C, D** diakinesis of *C. ornatus*, Ubatuba population, arrow – bivalent with large CMA_3^+ block **E, F** diakinesis of *C. medius* **G, H** mitotic metaphase of *Phoneutria nigriventer*, 2n=28 **I, J** diakinesis of *Viracucha andicola* **K, L** diakinesis of *Enoploctenus cyclothorax*. Bar = 10 µm.

chromosomes (Fig. 1T), that confirm sex chromosome systems of the types X_1X_20 and $X_1X_2X_30$, respectively. In species with 28 chromosomes, two positively heteropycnotic bodies were observed in pachytene stage (Fig. 1B, F, J, N) and *V. andicola* exhibited three positive heteropycnotic bodies (Fig. 1R), identified as the sex chromosomes.

Ctenus ornatus presented interstitial and terminal CMA_3^+ bands (Fig. 2A). Nevertheless, the population of Ubatuba (São Paulo state) presented an additional large terminal CMA_3^+ block in a bivalent (Fig. 2C). In *C. medius* (Fig. 2E), *P. nigriventer* (Fig. 2G), *V. andicola* (Fig. 2I), and *E. cyclothorax* (Fig. 2K), all populations showed only CMA_3^+ terminal blocks. Karyotypes contained no $DAPI^+$ blocks (Fig. 2B, D, F, H, J, L).

The FISH revealed one bivalent with 18S rDNA site in *C. ornatus* (Fig. 3A), *C. medius* (Fig. 3B), *V. andicola* (Fig. 3D), and *E. cyclothorax* (Fig. 3E). *C. ornatus* presented size polymorphism of the 18S rDNA site (Fig. 3A-box). *P. nigriventer* showed three bivalents exhibiting 18S rDNA site; however, one of these bivalents presented site only in one chromosome (Fig. 3C).

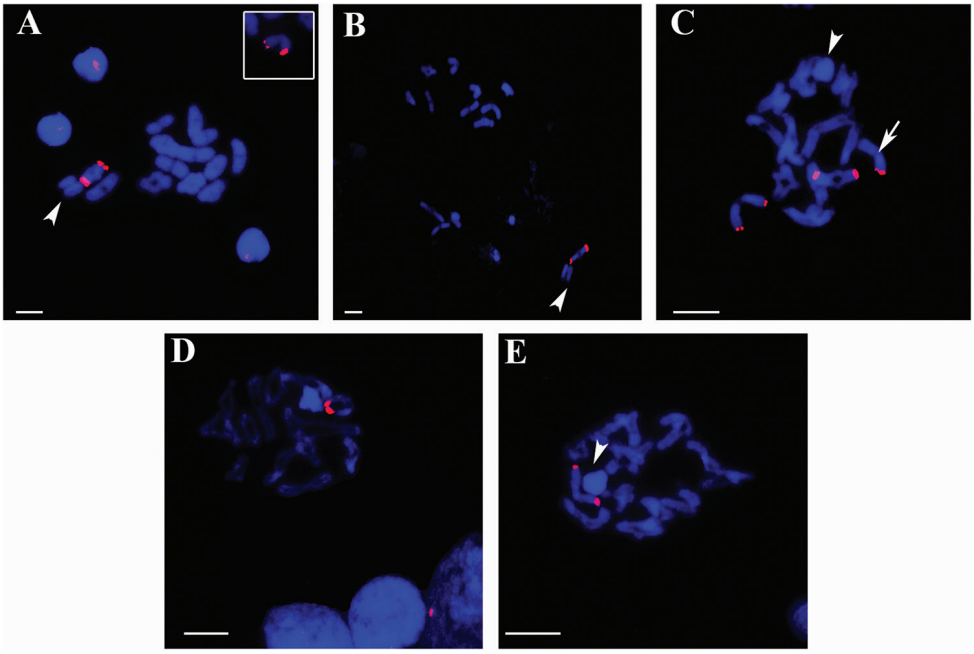

Figure 3. Ctenidae male meiotic cells, FISH with rDNA 18S probe. Arrowhead - sex chromosomes. **A** diakinesis of *Ctenus ornatus*: in the box the bivalent with size heteromorphism of 18S rDNA sites **B** diakinesis of *Ctenus medius* **C** diakinesis of *Phoneutria nigriventer*: arrow - bivalent with 18S rDNA sites in only one of the chromosomes **D** diplotene of *Viracucha andicola* **E** diplotene of *Enoploctenus cyclothorax*. Bar = 10 μm.

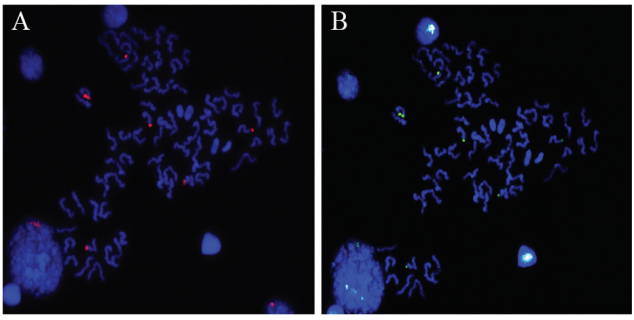

Figure 4. Chromosomes of *Ctenus ornatus*, Ubatuba/São Paulo state. **A** Metaphase II, FISH with rDNA 18S probe **B** sequential staining with DAPI/CMA_3 in the same metaphase II, showing association between sites of GC-rich heterochromatin and rDNA 18S regions. Note the presence of more than one metaphase II. Bar = 10 μm.

Metaphase II of *C. ornatus* from the Ubatuba population submitted to FISH and subsequently to CMA3/DAPI also revealed that CMA^+ sites with higher accumulation of GC-rich heterochromatin are co-localized to the sites carrying 18S rDNA (Fig. 4).

Discussion

The conventional analysis showed diploid number, chromosomal morphology, sex chromosome system and meiotic behavior of five Ctenidae species. The present study presents the first data for Acantheinae, increasing to four the number of ctenid subfamilies with cytogenetic data (Table 1), and the first cytogenetic study in *C. medius* and *E. cyclothorax*. In Ctenidae, the diploid number variation occurs basically due to the differences in SCS: species with 2n♂ = 28 exhibit a SCS of the type X_1X_20, whereas species with 2n♂ = 29 have the type $X_1X_2X_30$. Only *A. borellii* (Viridasiinae) presents 2n♂ = 22, with SCS of the type X_1X_20 (Chen 1999, Araujo et al. 2014, Kumar et al. 2016).

The parallel association between sex chromosomes during male meiosis is a common pattern observed in Entelegynae (Král et al. 2011; Araujo et al. 2012), and also found in Ctenidae (Chen 1999; Araujo et al. 2014; Kumar et al. 2016). Forman et al. (2013) observed absence of sex chromosome pairing in some plates of *Wadicosa fidelis*. They proposed that it might be due to chromosome preparation. A similar situation may have occurred in species analyzed in this study.

We observed two distinct distribution patterns of the GC-rich heterochromatin: (i) bands distributed in terminal regions of most chromosomes, as presented in *C. medius*, *P. nigriventer*, *E. cyclothorax* and *V. andicola*; and (ii) bands present in interstitial regions of most chromosomes, in addition to the terminal regions, as observed for *C. ornatus*. The first pattern could arise by dispersion of heterochromatin due to contact of chromosomes during their polarization of Rabl in mitosis or during bouquet orientation at the early prophase I as described by Schweizer and Loidl (1987). The second pattern could arise by occurrence of chromosomal rearrangements (Schweizer and Loidl 1987) or by spreading of the heterochromatin by transposable elements, as proposed for grasshopper (Rocha et al. 2015). Furthermore, despite the few species studied, GC-rich blocks seem to be common in entelegyne spiders (Araujo et al. 2005; Ramalho et al. 2008, Chemisquy et al. 2008). They were also found in Ctenidae species in the present study. The heterochromatin distribution also allowed to distinguish *C. ornatus* from Ubatuba population of other *C. ornatus* populations here analyzed.

The present study revealed a massive accumulation of GC-rich heterochromatin associated with 18S rDNA site in *C. ornatus* from Ubatuba. Association of GC-rich heterochromatin with NORs is common in many animal groups, for example in fishes (Ferro et al. 2001) and amphibians (Schmid 1980). In spiders, this association has been reported in *Nephilingys cruentata* (Araneidae) (Araujo et al. 2005).

Another characteristic observed in *C. ornatus* was the size heteromorphism of 18S rDNA sites. This can be explained by unequal crossing, which causes a greater accumulation of rDNA cistrons in one of the homologous chromosomes, as described by Ferro et al. (2001) and Teribele et al. (2008) in fish species. A similar situation may have occurred in *P. nigriventer*, very small 18S rDNA sites could exhibit low fluorescence, making detection difficult.

In Ctenidae, NOR in one bivalent seems to be the most commonly observed pattern. Only *P. nigriventer* presented more rDNA sites. This finding differs from Araujo et al. (2014), who observed only one chromosome pair carrying NOR in the same species using the silver nitrate impregnation that identifies only transcriptionally active sites. Specimens of *V. andicola* showed a single NOR as revealed by the FISH analysis. By contrast, the data exhibited by Araujo et al. (2014) showed NORs in two chromosome pairs, which could indicate an interpopulation variation, however the authors analyzed only one specimen, which hinders a more accurate study.

The present study brings new cytogenetic information and first FISH data for Ctenidae providing valuable contribution to the knowledge on karyotypes in this family.

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RESEARCH ARTICLE

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A chromosomal analysis of *Nepa cinerea* Linnaeus, 1758 and *Ranatra linearis* (Linnaeus, 1758) (Heteroptera, Nepidae)

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Abstract

An account is given of the karyotypes and male meiosis of the Water Scorpion *Nepa cinerea* Linnaeus, 1758 and the Water Stick Insect *Ranatra linearis* (Linnaeus, 1758) (Heteroptera, Nepomorpha, Nepidae). A number of different approaches and techniques were tried: the employment of both male and female gonads and mid-guts as the sources of chromosomes, squash and air-drying methods for chromosome preparations, C-banding and fluorescence *in situ* hybridization (FISH) for chromosome study. We found that *N. cinerea* had a karyotype comprising 14 pairs of autosomes and a multiple sex chromosome system, which is $X_1X_2X_3X_4Y$ (♂) / $X_1X_1X_2X_2X_3X_3X_4X_4$ (♀), whereas *R. linearis* had a karyotype comprising 19 pairs of autosomes and a multiple sex chromosome system $X_1X_2X_3X_4Y$ (♂) / $X_1X_1X_2X_2X_3X_3X_4X_4$ (♀). In both *N. cinerea* and *R. linearis*, the autosomes formed chiasmate bivalents in spermatogenesis, and the sex chromosome univalents divided during the first meiotic division and segregated during the second one suggesting thus a post-reductional type of behaviour. These results confirm and amplify those of Steopoe (1925, 1927, 1931, 1932) but are inconsistent with those of other researchers. C-banding appeared helpful in pairing up the autosomes for karyotype assembly; however in *R. linearis* the chromosomes were much more uniform in size and general appearance than in *N. cinerea*. FISH for 18S ribosomal DNA

(major rDNA) revealed hybridization signals on two of the five sex chromosomes in *N. cinerea*. In *R. linearis*, rDNA location was less obvious than in *N. cinerea*; however it is suggested to be similar. We have detected the presence of the canonical "insect" $(TTAGG)_n$ telomeric repeat in chromosomes of these species. This is the first application of C-banding and FISH in the family Nepidae.

Keywords

Karyotype, C-banding, $(TTAGG)_n$, 18S rDNA, FISH, male meiosis, *Nepa cinerea*, *Ranatra linearis*, Nepomorpha, Heteroptera

Introduction

Heteropteran cytogenetics was reviewed by Ueshima (1979). He listed data on nine species of the water bug family Nepidae – three *Laccotrephes* Stål, 1866, one *Nepa* Linnaeus, 1758 (*N. cinerea* Linnaeus, 1758, listed as *N. rubra* Linnaeus, 1758) and five *Ranatra* Fabricius, 1790, including *R. linearis* (Linnaeus, 1758). The chromosome complements in males range from diploid numbers (2n) of 33 (*Nepa cinerea*) to 46 (*Ranatra chinensis* Mayer, 1865), and the sex chromosomes are listed as either XY or X_nY, or in one case X(0). Although the different sex chromosome systems are often recorded from different species, this is not always the case. Thus, *R. chinensis* is listed as having 2n = 46, comprising 44 autosomes plus XY sex chromosomes by Shikata (1949), but also as having 2n = 43 including 38 autosomes plus $X_1X_2X_3X_4Y$ sex chromosomes by Ueshima (1979), using his own data. *Nepa cinerea* is listed by Spaul (1922) as having 2n = 35 (♂) with a simple sex chromosome system X(0) and 36 (♀), while the more extensive studies by Steopoe (1925, 1931, 1932) led to a male karyotype with 33 chromosomes, including 14 pairs of autosomes and $X_1X_2X_3X_4Y$ sex chromosomes, a result supported by Halkka (1956). The only data listed for *Ranatra linearis* by Ueshima (1979) are from Steopoe (1927), who gives the chromosome complement as 2n = 43 (♂), including 19 pairs of autosomes and $X_1X_2X_3X_4Y$ sex chromosomes. However, more recent work by Arefyev and Devyatkin (1988) based on the cell suspension preparation describes the complement as 2n = 46 (♂), postulating a simple sex chromosome system XY without any special arguments. Thus, there is either great variation between different populations of the above mentioned species, or some of the data might not be properly interpreted.

The early work on bugs was done using serial sections and this is also true of the objects of the present paper, the Water Scorpion *Nepa cinerea* and the Water Stick Insect *Ranatra linearis*. This technique can give very precise information on the orientation of the chromosomes in dividing nuclei and of the nuclei themselves within the tissues or organs (usually testes), but is of limited value in determining the sizes and shapes of the various chromosomes. Steopoe's papers (1925, 1927, 1931) are particularly clear. For *N. cinerea* and *R. linearis* he shows both first and second male meiotic metaphases (MI and MII) with a ring of chromosomal elements round the edge of the metaphase plate, and a group of five chromosomes, arranged like the spots on a die, in the centre of the ring of chromosomes. The chromosome at the centre of this group is often the largest

one. It has been demonstrated that, in this type of metaphase plate, the ring of chromosomes is made up of autosome bivalents (MI) or autosome univalents (MII), whereas the chromosomes in the centre behave as univalents (MI) or form a pseudobivalent / pseudomultivalent (MII) (Ueshima 1979). A striking feature of Steopoe's work on both *Nepa* and *Ranatra* is that the median group of five appears much the same at both first and second meiotic metaphases. For this to be the case these chromosomes must be univalents and undergo an equational (mitotic) division during first meiosis. Steopoe interpreted these chromosomes as four X chromosomes assembled round a larger Y chromosome, and Halkka (1956) showed an early second anaphase in *N. cinerea* with one of the central elements moving to one pole and the other four to the other one. Neither Steopoe nor Halkka gave a female chromosome count, but for the system they describe to work, it has to be 2n = 36 in *Nepa cinerea* (as in Spaul 1922) and 2n = 46 in *Ranatra linearis*. Therefore, clear establishment of both male and female karyotypes should show which of the sex chromosome systems is present in these bugs.

The chromosomes in Heteroptera are holokinetic (Ueshima 1979). These chromosomes lack physical landmarks such as primary constrictions (the centromeres) and thus possess very few differentiating features. In recent years, different chromosome banding techniques (primarily C-, fluorochrome- and AgNOR-bandings) and Fluorescence *In Situ* Hybridization (FISH) have made it possible to get some chromosomal markers in karyotypes of Heteroptera (e.g., Grozeva et al. 2003, 2004, 2010, 2011, 2015, Angus et al. 2004, Waller and Angus 2005, Bressa et al. 2005, 2009, Angus 2006, Papeschi and Bressa 2006, Panzera et al. 2010, 2012, Poggio et al. 2011, 2012, 2013, 2014, Kuznetsova et al. 2012, 2015, Chirino et al. 2013, 2017, Chirino and Bressa 2014, Golub et al. 2015, 2016, Pita et al. 2016, Salanitro et al. 2017).

A prerequisite for good chromosome preparations is well spread cells with the chromosomes lying in one focal plane; however such cells are difficult to obtain using the squash method which is nowadays the most generally employed means of Heteroptera chromosome preparations. Besides, the use of this technique, which involves the placement of a cover slip over a tissue (usually testicular follicles) for flattening and spreading the chromosomes, can cause their damage and loss. Recently, a series of studies by Angus and co-authors (Angus et al. 2004, Waller and Angus 2005, Angus 2006) showed that an air-drying method combined with C-banding is a useful means of revealing cytogenetic markers allowing assembly of karyotypes from holokinetic chromosomes of several aquatic species, specifically of *Notonecta* Linnaeus, 1758 and *Corixa* Geoffroy, 1762 (Nepomorpha, Notonectidae and Corixidae, correspondingly).

In the present work we performed a detailed analysis of the karyotypes and male meiosis in *Nepa cinerea* and *Ranatra linearis* based on chromosome slides prepared from male and female gonads and mid-guts by air-drying and squash methods, including chromosome lengths and patterns of C-band distribution. Additionally, the work included the examination of the number and chromosomal location of major rDNA clusters and molecular structure of telomeres by FISH with 18S rDNA and the "insect" telomeric $(TTAGG)_n$ probes. This is the first employment of C-banding and FISH for the water bug family Nepidae.

Material and methods

The localities (English and Bulgarian) from which the bugs were collected are given in Table 1.

The air-drying method of chromosome preparations and that of C-banding are as described by Angus et al. (2004). The living tissue was treated for 12.5 min in both the colchicine solution (0.1%) and the 0.5-isotonic KCl solution. C-banding was carried out on the 2-day old slides. Where slides had been Giemsa-stained and photographed under oil immersion, the oil was removed by immersion in xylene (2 changes, 5 min each) followed by 5 min in absolute ethanol. The slides were then destained by immersion in 2 × SSC for 10 min at 60°C and rinsed in unbuffered distilled water before the barium hydroxide treatment (about 8 min in saturated $Ba(OH)_2$ solution at about 23°C, room temperature). The destaining in 2 × SSC may be unnecessary as R. Angus (unpublished data) now routinely C-bands Giemsa-stained slides of Coleoptera chromosomes, applying the $Ba(OH)_2$ treatment to the slides once they have dried after immersion in absolute ethanol. The squash method of chromosome preparations and FISH procedure with 18S rDNA and $(TTAGG)_n$ probes were performed as described previously (Grozeva et al. 2011, 2015, Kuznetsova et al. 2012, 2015).

Giemsa stained and C-banded preparations were analysed under a Leitz Orthoplan microscope and photographed using a Wild MPS 51 camera and a Wild Photautomat MPS 45 with Kodak HQ high-contrast microfilm. Photographs were printed at 3000 × magnification, and then scanned into a computer where further manipulation and analysis of the images were done using Adobe Photoshop.

FISH preparations were analysed under a Leica DM 6000 B microscope, and images were acquired using a Leica DFC 345 FX camera and Leica Application Suite 3.7 software with an Image Overlay module.

The specimens from whom the chromosome preparations have been obtained are housed in R. Angus' collection (Natural History Museum, London, UK) and at the Institute of Biodiversity and Ecosystem Research, BAS (Sofia, Bulgaria), correspondingly.

Table 1. Localities where *Nepa cinerea* and *Ranatra linearis* were collected.

Species	Localities and number of specimens analysed
Nepa cinerea	UK, Surrey: Ash, Lakeside Park (1♂, 1♀) 51.26°N 0.73°W
	Middlesex: Staines Moor (1 ♀) 51.52°N 0.52°W
	West Norfolk: Thompson Common (1♂) 52.52°N 0.82°E
	Bulgaria, Sofia: artificial lake in a park (1♂ juv.) 42.66°N 23.31°E
Ranatra linearis	UK, East Sussex: Pevensey Level (2 ♂♂) 50.81°N, 0.34°E
	Surrey: Runnymede, Langham Pond (2♀♀) 51.44°N, 0.56°W
	Bulgaria: Srebarna lake, (1♂ juv.) 44.10°N, 27.06°E

Results

C-banding

Nepa cinerea, 2n (♂ / ♀) = 33 / 36 (14 AA + $X_1X_2X_3X_4Y$ / $X_1X_1X_2X_2X_3X_3X_4X_4$)

Male and female mitotic karyotypes (karyograms) are shown in Fig. 1a–e. First male meiosis is shown in Figs 2a–d and 3a, b, and second male meiosis is shown in Fig. 3c, d. Relative Chromosome Lengths (RCL, the length of each chromosome expressed as a percentage of the total haploid autosome length in the nucleus) are given in Table 2. Comparison of the C-banded karyotypes shows that the female (Fig. 1a, e) has four pairs of chromosomes which appear to be matched by single unpaired ones in the male (Fig. 1b-d), which also has a further large single chromosome. The large chromosomes, which are unpaired in the male but paired in the female, must be two of the four X chromosomes. The remaining unpaired chromosomes in the male are the large Y chromosome which has no counterpart in the female karyotype, and the two smaller ones which are taken as X_3 and X_4, but they are so small (the smallest chromosomes of the complement) that, on the present material, it is not possible to demonstrate that they are of the same or different sizes.

C-banding shows that the larger autosomes (pairs 1 – 4) have a distinct C-band at each end, but with some variation, possibly due to inadequacies of the C-banding method (Fig. 1). The C-banding pattern of the medium-sized autosomes (5 – 11) is variable, but the C-bands tend to be concentrated at one end and in the smaller autosomes, they are probably absent. Of the sex chromosomes, X_1, X_2 and Y have a strong C-band at one end while X_3 and X_4 have no clear banding. The banding of the autosomes in Fig. 1e reflects only partial success with the C-banding protocol.

The group of five chromosomes shown by Steopoe (1925, 1931, 1932) and Halkka (1956) as lying in the middle of the meiotic metaphase plate is very clear at second metaphase (Fig. 3c, d), but the position of these chromosomes is less distinctive at the first metaphase and diakinesis (Figs 2, 3a, b). It should be noted that the preparations figured here were made following colchicine treatment, which disrupts spindle formation, as well as cell-inflation by hypotonic saline. It is therefore not surprising that the orientation of the chromosomes is less clear than in the earlier work, which was done by serial sectioning. The arrangement of the sex chromosomes in metaphase plates of both divisions of meiosis shows that, while the autosomal bivalents separate and the homologs move to opposite poles of the spindle during first division, the sex chromosomes undergo an equational division at this stage. Thus, at second metaphase there is a ring of double-stranded autosomes which undergo an equational division and a group of single-stranded sex chromosomes, which segregate into four X chromosomes going to one pole and the Y chromosome which goes to the other one (the sex chromosomes post-reduction).

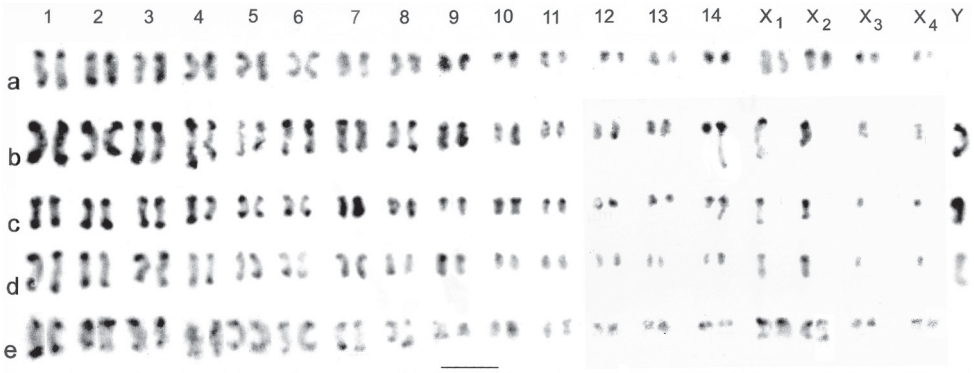

Figure 1. *Nepa cinerea*, C-banded mitotic chromosomes arranged as karyotypes. **a** ♀, ovary, Staines Moor **b** ♂, testis, Thompson Common **c, d** ♂, testis, Ash **e** ♀, mid-gut, Ash. Bar = 5 µm.

Table 2. Relative Lengths of *Nepa cinerea* chromosomes (measured in 3 males and 1 female).

Chromosome	RCL: mean (95% confidence intervals by t-test)	Number of measured cells
1	11.83 (10.86–12.80)	10
2	10.33 (9.79–10.87)	10
3	10.16 (9.68–10.64)	10
4	9.84 (9.28–10.40)	10
5	9.20 (8.16–10.24)	10
6	8.86 (8.13–9.59)	10
7	7.93 (7.61–8.25)	10
8	6.71 (6.10–7.32)	10
9	5.44 (4.76–6.12)	10
10	4.93 (4.32–5.54)	10
11	4.67 (4.29–5.05)	10
12	3.34 (2.81–3.37)	10
13	3.26 (2.75–3.77)	10
14	3.22 (2.56–3.88)	10
X_1	7.39 (6.12–8.65)	7
X_2	6.17 (5.57–6.77)	7
X_3	3.14 (2.57–3.72)	7
X_4	2.59 (1.92–3.25)	7
Y	9.80 (8.06–11.54)	3

Ranatra linearis, 2n (♂ / ♀) = 43 / 46 (19 AA + $X_1X_2X_3X_4Y$ / $X_1X_1X_2X_2X_3X_3X_4X_4$)

Male and female mitotic karyotypes (karyograms) are shown in Fig. 4a–c. First metaphase of meiosis is shown in Fig. 5a–c and second metaphase in Fig. 5d, e. The karyotype includes 2n = 43 (♂) and 46 (♀). *R. linearis* has five more pairs of autosomes than *N. cinerea*, and the chromosomes are mostly smaller. The differences in chromosome

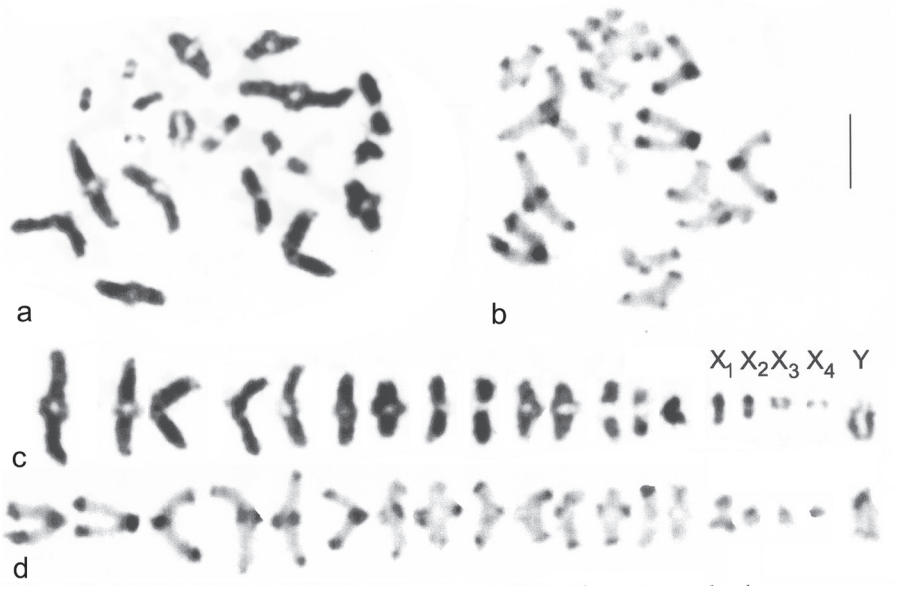

Figure 2. *Nepa cinerea*, ♂, Ash, first meiotic diakinesis/metaphase I from testis. **a, c** Giemsa-stained **b, d** C-banded. **a, b** nuclei as found **c, d** the same nuclei plated out and with the sex chromosomes labelled. Bar = 5 µm.

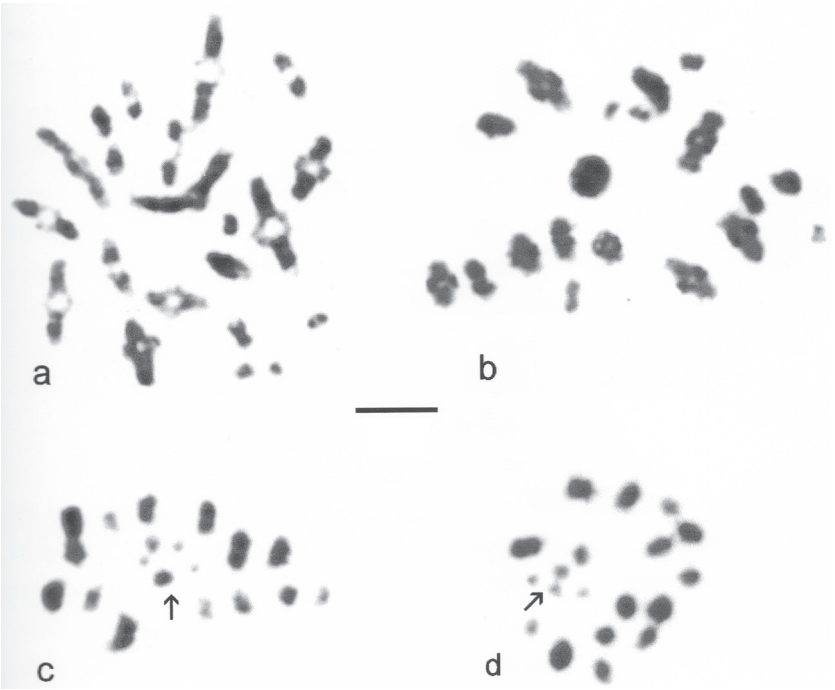

Figure 3. *Nepa cinerea*, ♂, Ash, first and second meiotic metaphases. **a, b** metaphase I **c, d** metaphase II. Sex chromosomes arrowed in **c, d**. Bar = 5 µm.

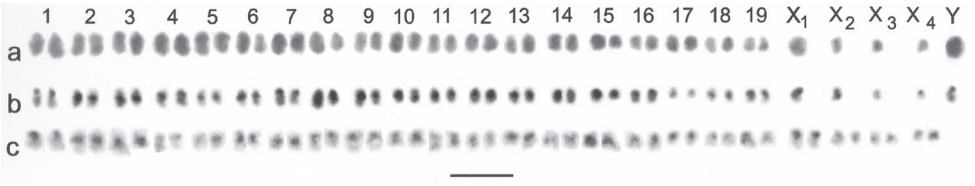

Figure 4. *Ranatra linearis*, mitotic chromosomes arranged as karyotypes. **a, b** ♂, Pevensey, testis **a** Giemsa-stained **b** the same nucleus, C-banded **c** ♀, Runnymede, mid-gut, C-banded. Bar = 5 µm.

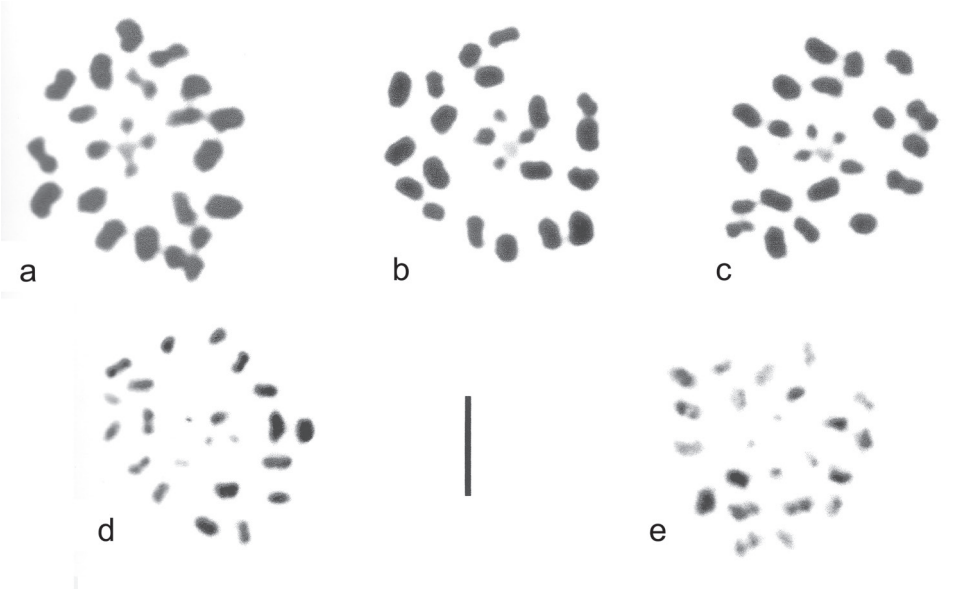

Figure 5. *Ranatra linearis*, ♂, Pevensey, meiosis **a–c** metaphase I **d, e** metaphase II. The central group of five chromosomes is very clear at first metaphase, but they have become displaced at metaphase II, especially in **e**. Bar = 5 µm.

length along the karyotype are less obvious than in *Nepa*, making the assembly of a karyotype more difficult. C-banding shows that nearly all the autosomes have one C-band at median, subterminal or terminal postions. Comparison of the karyotypes shown in Fig. 4a, b (unbanded and C-banded male mitotic chromosomes of the same nucleus) and Fig. 4c (C-banded female mitotic chromosomes) shows how C-banding can reveal more of the shape of individual chromosomes. Thus, the unbanded chromosomes tend to appear as elliptical masses but once C-banded they appear more rod-like. The pattern of sex chromosomes ($X_1X_2X_3X_4Y$), and their behaviour during the two divisions of meiosis, is the same as in *Nepa cinerea*. The arrangement pattern of the central group of five sex chromosomes is particularly clear in cells at metaphase I (Fig. 5a–c) and also in one cell at metaphase II (Fig. 5d), but they have been more disrupted by colchicine treatment and become displaced in other metaphases II (Fig. 5e). The general appear-

ance of these sex chromosomes at the both metaphases is very similar, like the spots on a die. A similar resemblance of the general appearance of the sex chromosomes during first and second metaphases of meiosis has been shown by Suja et al. (2000) for species of the heteropteran families Pentatomidae, Pyrrhocoridae and Coreidae.

FISH mapping of 18S rDNA and TTAGG telomeric repeats

Figure 6a–c presents an example of the $(TTAGG)_n$ telomeric repeat distribution and major rDNA location at mitotic metaphase of a *N. cinerea* male (a) and at first metaphase (MI) of a *R. linearis* male (b, c), both males originating from Bulgaria. It is evident from the figure that the telomeric probe labels the ends of several chromosomes in both species indicating thus the presence of canonical pentameric insect telomeric repeats TTAGG in their genomes. It is interesting that in *N. cinerea* some of the larger chromosomes (with the heaviest C-bands) do not appear to show the telomeric signals. In *R. linearis*, with the meiotic metaphases, it does not seem possible to demonstrate with confidence the localization of the telomeric signals. In *N. cinerea*, FISH experiments with the 18S rDNA probe showed sharp and intense hybridization signals on two chromosomes, the signals being located at interstitial position on the larger chromosome and at terminal region on the smaller one. Since these chromosomes differ in size and rDNA clusters location, they

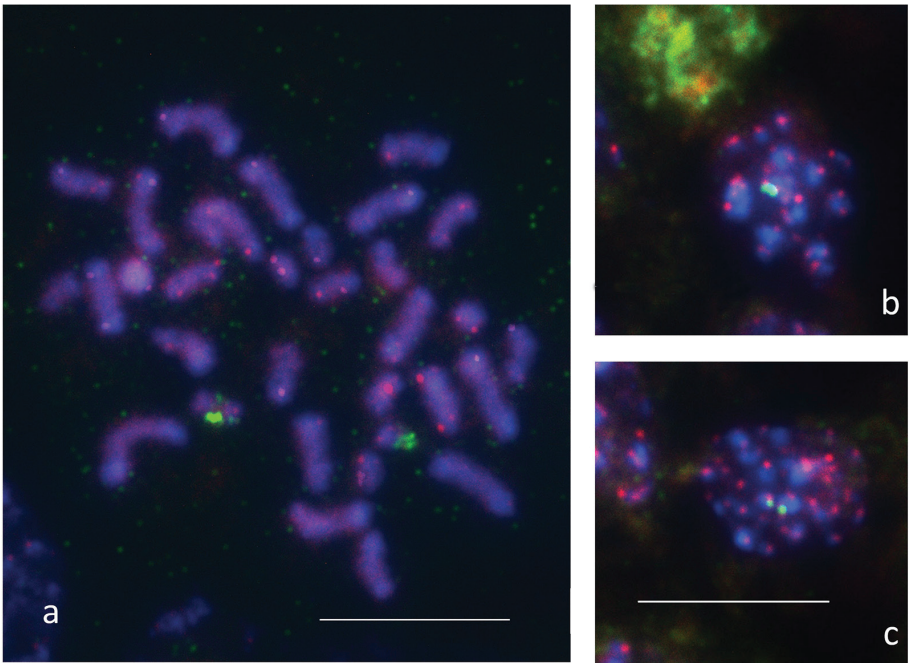

Figure 6. FISH with telomeric $(TTAGG)_n$ (red signals) and 18S rDNA (green signals) probes on mitotic chromosomes of *Nepa cinerea* (**a**) and meiotic chromosomes of *Ranatra linearis* (**b, c**). Two small signals (**c**) are united into one large signal (**b**). Bar = 10μm.

are most likely either the X chromosomes (two of the four) or an X and the Y chromosomes. In *R. linearis*, the 18s rDNA probe identified two hybridization signals associated with two chromosomal elements of different size in the meiotic cells analysed (Fig. 6c). Based on the meiotic stages observed, we failed to determine the precise location of rDNA sites. Nevertheless, given that they are situated at one end of the chromosomal units, these are most likely univalents (i.e., sex chromosomes) rather than bivalents.

Discussion

One of the first results to come from this work is to show how, in *Nepa cinerea* and *Ranatra linearis* from the Nepidae, as in species of other nepomorphan families, Corixidae and Notonectidae (Angus et al. 2004, Waller and Angus 2005, Angus 2006), the use of hypotonic-inflation and air-drying technique followed by C-banding, allows realistic karyotypes to be assembled. With holokinetic chromosomes, the only morphological characteristic available for karyotype production is chromosome length. Given that in most cases more than one pair of chromosomes will be more or less the same length, and that chromosomes show irregular condensation in individual preparations, it is not possible to produce convincing karyotypes using length alone. In Nepidae, the situation is made even worse because of the elliptical or blob-like appearance shown by many of the unbanded chromosomes. C-banding has the advantage of not only showing heterochromatic bands where they are present, but also of clearing the blob-like heavy staining of the chromosomes, so their actual shape becomes apparent. In *Nepa cinerea* the chromosomes are fairly long, the longer ones about 3μm in length, with C-bands at each end. These chromosomes tend to look distinctive, so that they can be paired up and karyotypes may be assembled with a fair degree of confidence, despite some ambiguity. The results from *N. cinerea* are useful as they show that the chromosome complement and sex chromosome arrangement described by Steopoe (1925, 1931, 1932) are in accordance with the cytogenetic description presented herein.

Spaul (1922) appears to have been mistaken about the sex chromosomes in this species, despite the apparent clarity of his drawings. However, Spaul is the only person to have published the female complement (2n = 36) – correctly. In his earlier papers, Steopoe (1925, 1931) used haematoxylin stained preparations and was concerned with the association between the chromosomes and the "plasmosome" (nucleolus) during meiosis. In particular, he thought that the association between the nucleolus and the group of five medially positioned sex chromosomes was the mechanism behind their positioning on the metaphase plate. Later, when he used Feulgen staining to show that the chromosomes and the nucleolus were chemically different (DNA and RNA) he attached less importance to this association (Steopoe 1932). The more recent observations of, e.g., Ueshima (1979), Nokkala (1986) and Kuznetsova et al. (2011, see also references therein) showed that in both meiotic metaphase plates involving the holokinetic chromosomes of heteropterans the autosomal bivalents/univalents (MI/MII) tend to form a ring round the edge of the plates whereas sex univalents and pseu-

dobivalents/pseudomultivalents occupy the centre, which accounts very well for the arrangement shown by both *N. cinerea* and *R. linearis*.

The chromosomes of *R. linearis*, though amenable to the protocols used in this study, are both smaller and more numerous than those of *N. cinerea*, and the karyotype suggested has to be more tentative. However, the chromosome complement, with 19 pairs of autosomes, and sex chromosomes as in *N. cinerea*, is clear. One piece of new information in this study is the female karyotype of *R. linearis*, with three more chromosomes than the male, as in *N. cinerea*. The multiple sex chromosome system $X_1X_2X_3X_4Y$ / $X_1X_1X_2X_2X_3X_3X_4X_4$ (male/female) found in these nepids stands in sharp contrast to the straightforward XY system found in Notonectidae and Corixidae (Ueshima 1979, Angus et al. 2004, Waller and Angus 2005, Bressa and Papeschi 2007). However, the multiple system may have originated from fragmentation of an original single (but large) X chromosome. Since the chromosomes are holokinetic, fragmentation does not result in loss of chromosome bits during cell division. The multiple sex chromosome systems, being found in species of Nepoidea and Ochteroidea, should be considered as derived characters within Nepomorpha (Bressa and Papeschi 2007).

One somewhat curious aspect of published work on the chromosomes of both *Nepa* and *Ranatra* is the two parallel views on the number of autosomes and sex chromosome mechanisms. Thus Spaul (1922) suggested diploid numbers of 35 (♂) and 36 (♀) for *N. cinerea*, with X(0) sex chromosome mechanism. For *R. chinensis*, Shikata (1949) reported the male complement with 46 chromosomes, 22 pairs of autosomes and XY sex chromosomes, but Ueshima (1979) claimed it had 43 chromosomes in the male, and sex chromosomes as described here for *R. linearis*, i.e., $X_1X_2X_3X_4Y$. The final twist to this tale comes from Arefyev and Devyatkin (1988), who report a complement of 46 chromosomes, including XY sex chromosomes, for male *R. linearis*. Sadly, they give no figure. It is at first sight impossible to reconcile these conflicting accounts. However, the detailed study of spermatogenesis in *N. cinerea* by Halkka (1956) may offer an explanation. Halkka observed that the division of the centrioles took place rapidly and early in the meiotic cycle and in some cases led to irregularities in chromosome division, with the production of polyploid and aneuploid spermatids. All previous work has been on testes, except, perhaps, for that of Arefyev and Devyatkin (1988) who did not know which tissues they were using as chromosome sources. However, in our study some karyotypes are from mid-gut cells, not subject to irregularities in spermatogenesis, so the results may be taken as correct.

A summary of all information on chromosome complements in *N. cinerea* and *R. linearis* derived from different studies conducted at different times by different investigators is presented in Table 3.

Another important result of this work is to show that the major rDNA loci are located on the sex chromosomes of *N. cinerea* and most probably also of *R. linearis* and that the ends of their chromosomes, the telomeres are composed of the pentanuceotide repeats TTAGG. These are the first data for the family Nepidae. In Heteroptera, there is a wide variation of major rDNA location: on different pairs of autosomes, on one or two sex chromosomes or on both autosomes and sex chromosomes, the differences

Table 3. A summary of data on karyotypes in *Nepa cinerea* and *Ranatra linearis.*

Taxon	Diploid	Haploid	References
Nepinae			
Nepa cinerea	35 ♂	17AA + X(0)	Spaul 1922
	36 ♀		
	33 ♂	14AA + $X_1X_2X_3X_4Y$*	Steopoe 1925, 1931, 1932
	33 ♂	14AA + $X_1X_2X_3X_4Y$*	Halkka 1956
	33 ♂	14AA + $X_1X_2X_3X_4Y$	Present study
	36 ♀	14AA + $X_1X_1X_2X_2X_3X_3X_4X_4$	
Ranatrinae			
R. linearis	43 ♂	19AA + $X_1X_2X_3X_4Y$	Steopoe 1927
	46 ♂	22AA + XY	Arefyev and Devyatkin 1988
	43 ♂	19AA + $X_1X_2X_3X_4Y$	Present study
	36 ♀	19AA + $X_1X_1X_2X_2X_3X_3X_4X_4$	

*In Ueshima (1979) haploid complement of this species was erroneously presented as 19AA + $X_1X_2X_3X_4Y$

being sometimes observed even between closely related, congeneric species (reviewed in Grozeva et al. 2015). Likewise, this is true of Nepomorpha, where in the two previously studied genera, *Belostoma* Latreille, 1807 and *Lethocerus* Mayr, 1853 (Belostomatidae), some species have 18S rRNA genes on autosomes while others on sex chromosomes (Papeschi and Bressa 2006, Kuznetsova et al. 2012, Chirino et al. 2013, Chirino and Bressa 2014). The data currently available are still so scarce and limited in their taxonomic representativeness that any speculation would be highly premature.

The TTAGG tandem sequence repeat found in our study in *N. cinerea* and *R. linearis* is considered the most typical and ancestral telomeric motif within the class (Sahara et al. 1999, Frydrychová et al. 2004, Vítková et al. 2005, Lukhtanov and Kuznetsova 2010, Chirino et al. 2017). Despite the widespread distribution of the $(TTAGG)_n$ motif among insects, it is not universally present in each order. For example, the huge order Coleoptera includes both TTAGG-positive and TTAGG-negative species, which has been interpreted as the multiple (at least eight times) loss of the initial telomeric sequence during beetle evolution (Frydrychová and Marec 2002, Mravinac et al. 2011). A similar heterogeneity is clearly exhibited also by Heteroptera with some species showing evidence for canonical telomeres and others not. The order comprises 7 infraorders and 40,000 species (Weirauch and Schuh 2011). The studies of telomeric DNA sequences were limited to 25 species, 17 genera and 9 families in the infraorders Nepomorpha (the families Belostomatidae and Nepidae; Kuznetsova et al. 2012, Chirino et al. 2017, present study), Gerromorpha (Gerridae; Mason et al. 2016), Cimicomorpha (Miridae, Cimicidae, Tingidae and Reduviidae; Frydrychová et al. 2004, Grozeva et al. 2011, Golub et al. 2015, Pita et al. 2016) and Pentatomomorpha (Pyrrhocoridae, Pentatomidae; Frydrychová et al. 2004, Grozeva et al. 2011). The $(TTAGG)_n$ telomeric sequence – according to our present knowledge – is present in both more basal infraorders Nepomorpha and Gerromorpha. Likewise, the $(TTAGG)_n$ motif is present in a sister to Heteroptera suborder Coleorrhyncha (Kuznetsova et al.

2015) and in several genera of Sternorrhyncha and Auchenorrhyncha (see for references Kuznetsova et al. 2015 and Pita et al. 2016). This indicates that it was most likely the ancestral telomere repeat sequence of Hemiptera as a whole. On the other hand, the ancestral motif $(TTAGG)_n$ was suggested to be lost in the early evolution of the evolutionarily derived heteropteran lineage composed by the sister infraorders Cimicomorpha and Pentatomomorpha being secondarily replaced by another motif or an alternative telomerase-independent mechanism of telomere maintenance (Frydrychová et al. 2004, Lukhtanov and Kuznetsova 2010, Mason et al. 2016). In all previously checked representatives of the families Miridae, Cimicidae, Tingidae, Pyrrhocoridae, and Pentatomidae the $(TTAGG)_n$ motif has not been found which supported well the above suggestion. Moreover, our dot-blot experiments have eliminated TTTTGGGG, TTGGGG, TTAGGC, TAACC, TTAGGG and TTTAGGG alternative variants as a potential replacement in tested TTAGG-negative species (Grozeva et al. 2011). Noteworthy in this context is a recent survey of sequenced genomes of several pentatomomorphan and cimicomorphan species confirming the lack of the TTAGG telomeric repeat and allowing suggestion that these groups have a defective version of telomerase gene (Mason et al. 2016).

However, a recent study of Pita et al. (2016) discovered unexpectedly the putative ancestral "insect" motif in the cimicomorphan family Reduviidae, namely in the youngest reduviid subfamily Triatominae, casting doubt on the above hypothesis since, according to the authors' belief, "a new acquisition of the ancestral telomeric repeat in this recent evolutionary group is unlikely". Moreover, the postulated lack of the $(TTAGG)_n$ detection in Cimicomorpha and Pentatomomorpha, by their hypothesis, "is due to a methodological problem of the telomeric probe rather than a loss process during their evolution". We can not unconditionally agree with this view since in our studies, at least, the simultaneous labelling with the $(TTAGG)_n$ probe resulted in either a clearly defined or no FISH reaction in different species involved in the same experiment. To be sure, the absence of readable FISH signals in the particular taxa is not coincidental. One possibility is that in these taxa the TTAGG repeats are present but could not be localized by FISH due to their exclusively low amounts. It is our opinion that there still remains much work toward elucidating the problem and verifying the above hypotheses.

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SHORT COMMUNICATION

COMPARATIVE Cytogenetics
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Contribution to the cytogenetics of Kuwaniini scale insects (Homoptera, Coccinea, Margarodidae s.l.)

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Abstract

Jansenus burgeri Foldi, 1997 (Margarodidae s.l., Xylococcinae, Kuwaniini) was studied cytogenetically for the first time. It was shown that the species reproduces bisexually, displays XX/X(0) sex chromosome system and 2n=6/5 (female/male) including two pairs of long autosomes and a pair of shorter X-chromosomes in female. The chromosome complement, adult female morphology and the life cycle of *J. burgeri* are illustrated. The cytogenetic data are in fact the first ones for Kuwaniini scale insects, because *Kuwania oligostigma* De Lotto, 1959 briefly cytogenetically studied by Hughes-Schrader (1963), in my opinion, may be excluded from the genus *Kuwania* Cockerell, 1903 and the tribe Kuwaniini, since this species shows aberrant morphological characters, specifically the total absence of abdominal spiracles and the presence of tubular ducts.

Keywords

Jansenus burgeri, scale insects, morphology, life cycle, karyotype, sex chromosome system

The tribe Kuwaniini MacGillivray, 1921 (Margarodidae s.l., Xylococcinae), which currently comprises four nominal genera (Wu and Nan 2012), has not been explored cytogenetically up to the present excluding the brief note (without photographs) of Hughes-Schrader (1963) on chromosomal number (2n=16) in *Kuwania oligostigma* De Lotto, 1959, whose taxonomic position is questionable (see below). Two other genera of the tribe, *Neogreenia* MacGillivray, 1921 and monotypic *Neosteingelia* Morrison, 1927, have never been studied in terms of cytogenetics and reproductive biology. In 1997 Dr. Imre Foldi described from Thailand a peculiar new species and new monotypic genus, *Jansenus burgeri* Foldi, 1997, placed by him in the subfamily Xylococcinae without tribal attribution. Wu and Nan (2012) considered this genus as closely related to *Kuwania*, *Neogreenia* and *Neosteingelia*, and that conforms to my own view on the taxonomy of this group.

The exact type locality (in Thailand) for *Jansenus burgeri* was unknown and there have been no reports on new findings of this species since its original description. In June 2017 I was able to collect fresh material on this species during my expedition in Northern Laos (bank of Mekong, Pak Beng village, on stem under the bark of undetermined tree, adult females and larvae, guarded by ants, 14.VI.2017, I. Gavrilov-Zimin, collection number K 1385, preserved at Zoological Institute, Russian Academy of Sciences). The material was suitable both for morphological investigation (Fig. 1) and study of karyotype and chromosome system in this species. The chromosome counts were performed in young embryos of both sexes, squashed in a drop of lactoaceticorcein. *J. burgeri* was found to reproduce bisexually, have XX/X(0) chromosome system and 2n=6/5 (Fig. 2); diploid karyotype includes two pairs of long autosomes and a pair of shorter X-chromosomes in females while one X-chromosome in males. The mature female lays eggs before cleavage divisions in the white cottony wax sac suggesting thus the normal oviparity. As other Xylococcinae and some other Margarodidae s.l., *J. burgeri* has apodal cyst-like stages in its life cycle (Fig. 3).

Only a few species of Xylococcinae have been studied cytogenetically till now and all available data were published without photographs of the chromosomes. Thus, *Kuwania oligostigma* De Lotto, 1959 (tribe Kuwaniini) shows 2n=16 in adult female and bisexual reproduction (Hughes-Schrader 1963); the chromosome number in males and in embryos of both sexes were not studied. Meanwhile, the morphology of *K. oligostigma* is significantly different from that of other species of the genus and other genera of the tribe. According to the original morphological description and provided figure, *Kuwania oligostigma* has totally lost the abdominal spiracles (a unique situation for Xylococcinae!) and has tubular ducts in contrast to all other Kuwaninii. In my opinion, the species may be excluded from the tribe, but its correct taxonomic placement remains obscure for me.

Matsucoccus gallicolus Morrison, 1939 (Xylococcinae, Matsucoccini) was studied by Hughes-Schrader (1948) who reported for this species XX-X(0) chromosome system with multiple X chromosomes, i.e., 2n=28A+12X in females and 2n=28A+6X in males.

Nur (1980) studied gravid females of *Steingelia gorodetskia* Nasonov, 1908 (Xylococcinae, Steingeliini) and found that the species had the bisexual reproduction, XX-X(0) chromosome system and 2n=10 in females.

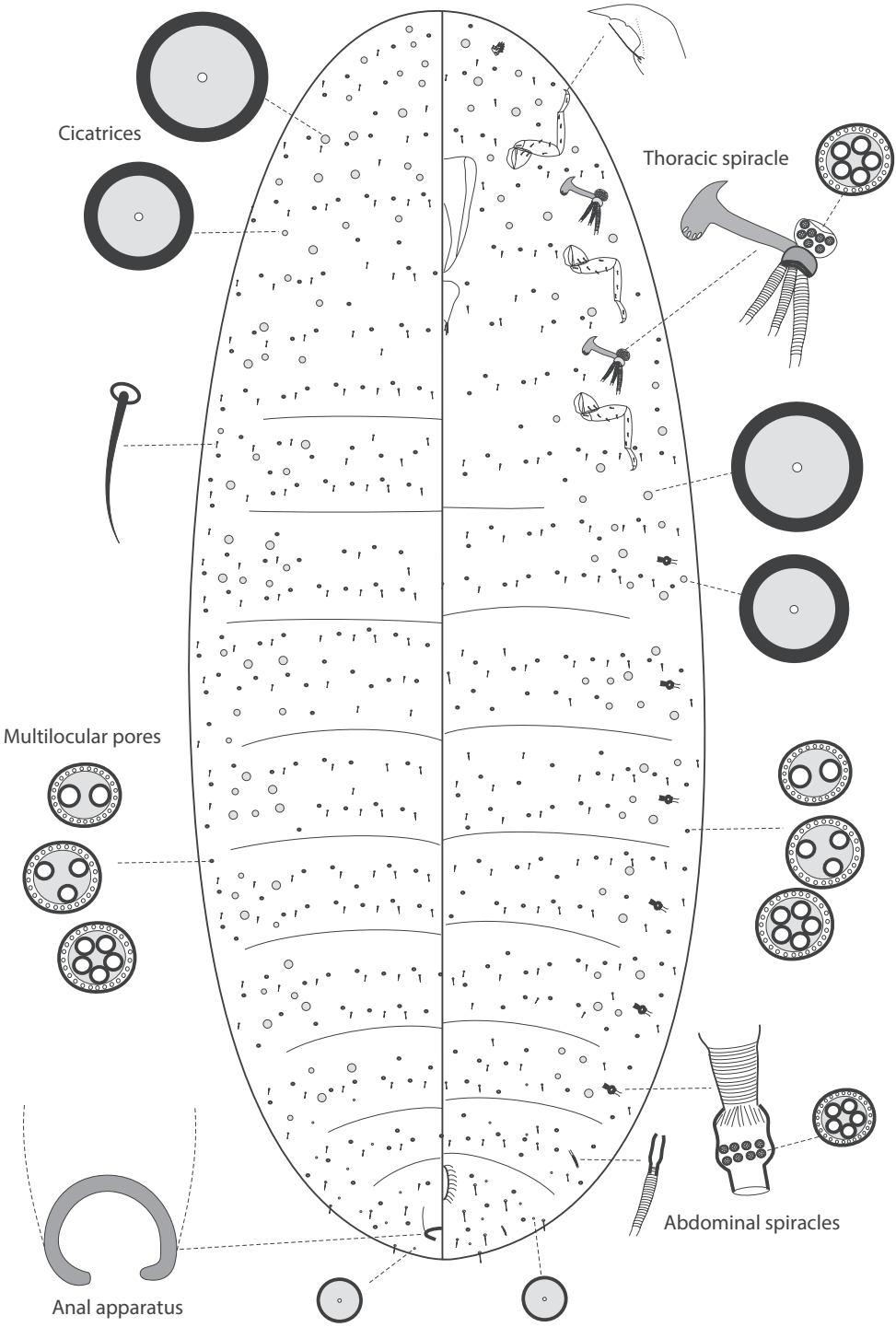

Figure 1. Morphology of adult female of *Jansenus burgeri* Foldi, 1997, Laos (Pak Beng).

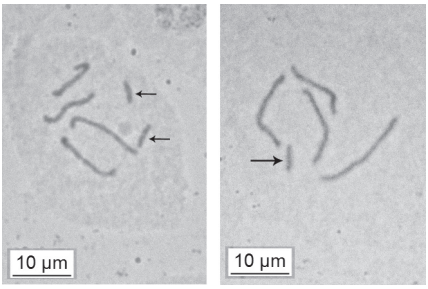

Figure 2. Embryonic cells of *Jansenus burgeri* Foldi, 1997 in female (2n=6) and male (2n=5) embryos; sex chromosomes are arrowed.

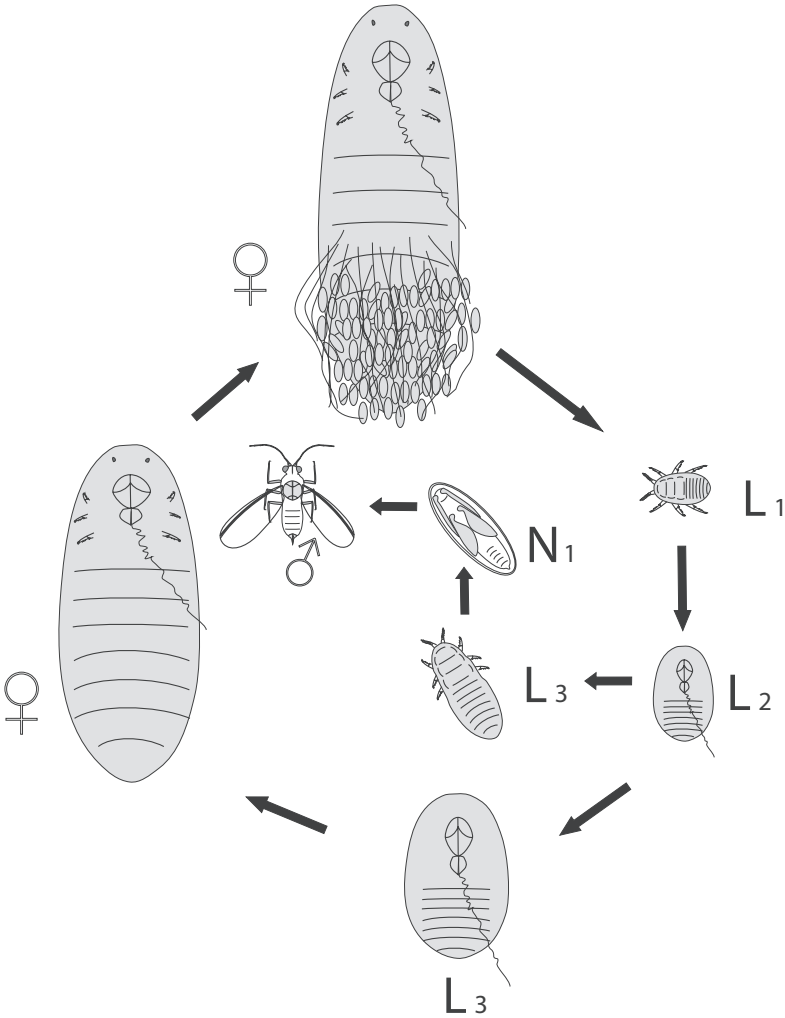

Figure 3. The life cycle of *Jansenus burgeri* Foldi, 1997; L_1–L_3 – first, second and third larval stages; N_1 – nymphal (preimaginal) stage with protoptera (wing buds) of male.

Thus, amongst the four Xylococcinae species studied so far, *Jansenus burgeri* shows the lowest chromosome number, 2n= 6. Of the other scale insects, the same number is known only in few species of the subfamily Monophlebinae (Orthezioidea, Margarodidae s.l.) and in some species of neococcids (superfamily Coccoidea) – see for review Gavrilov 2007.

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RESEARCH ARTICLE

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Contributions to the cytogenetics of the Neotropical fish fauna

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Abstract

Brazilian fish cytogenetics started as early as the seventies in three pioneering research groups, located at the Universidade Estadual Paulista (UNESP, Botucatu, SP), Universidade Federal de São Carlos (UFSCar, São Carlos, SP) and Universidade de São Paulo (USP, São Paulo, SP). Investigations that have been conducted in these groups led to the discovery of a huge chromosomal and genomic biodiversity among Neotropical fishes. Besides, they also provided the expansion of this research area, with the genesis of several other South American research groups, in view of a number of dissertations and doctoral theses developed over years. The current authors were encouraged to make their thesis catalog accessible from a public source, in order to share informations on the taxa and subject matter analyzed. Some of the key contributions to evolutionary fish cytogenetics are also being highligthed.

Keywords

Fish cytogenetics, Neotropical species, biodiversity, sex chromosomes, B chromosomes

Introduction

About 13,000 freshwater fish species are now recognized, 50% of them living in the Neotropical region (Reis et al. 2003), which emphasizes the significant parcel of the ichthyological diversity enclosed in this particular world region. Of course, this was one of the main reasons that attracted the attention of some Brazilian researchers, fostering the investigation on cytogenetics of Neotropical fishes.

Brazilian fish cytogenetics started in the early 70s, with three pioneering research groups located at the Universidade Estadual Paulista (UNESP, Botucatu, SP), Universidade Federal de São Carlos (UFSCar, São Carlos, SP) and Universidade de São Paulo (USP, São Paulo, SP). During this time, a lot of significant evolutionary and cytotaxonomic contributions were achieved, improving the knowledge on the biodiversity inside the rich Neotropical ichthyofauna.

The development of methodological approaches was certainly a key step for obtaining good chromosomal preparations and for improving fish cytogenetics. In this sense, the direct chromosome preparation from kidney cells, adapted in our early studies since 70s and recently revised (Bertollo et al. 2015), was largely utilized over years. In addition, the progressive application of conventional banding techniques (C, Ag-NORs, DAPI, CMA_3 staining), as well as more advanced methodologies combining cytogenetic and molecular procedures (chromosome mapping of DNA sequences by FISH, whole chromosome painting – WCP and comparative genomic hybridization – CGH) were essential tools in understanding the fish genome organization, particularly regarding to sex chromosome evolution and biodiversity investigations.

Although primarily and mainly devoted to freshwater species, the chromosomal analyses were also expanded to marine fishes, which is now the particular focus of some laboratories. From 1986 to now, successful biennual symposiums on fish cytogenetics are ongoing at different Brazilian regions. From some years ago, the discipline of genetics was also added to such meetings, with an expressive participation of professionals, students, as well as foreign invited researchers.

The catalogue of student theses, supervised in the Laboratory of Fish Cytogenetics of the Universidade Federal de São Carlos, comprises 42 doctoral theses and 52 master dissertations from 1981 to 2016. Informations about their corresponding students, taxa and matter subjects are available in the present communication, considering that not all the resulting data have been published. Theses/dissertations produced were assembled by taxonomic groups, according to Reis et al. (2003), regardless of their chronology. This criterion provides an overview of the different studied groups, considering that several families, genera and species have wider distribution and were subjected of more extensive investigations, being analyzed by different authors. The "taxa analyzed" item makes explicit when different populations, as well as different karyomorphs (karyotypes with distinct characteristics from each other) of a given species were investigated. The term “species group” was used for cases of specimens showing morphological similarities to a given valid species, but missing a proper taxonomic revision by the time they were studied.

Significantly, more than 20 research groups, nowadays located in different Brazilian regions, and also in Argentina, have emerged from such studies. These new researchers, along with those that have been emerged from the other pioneer laboratories, are now also engaged on fish chromosomal investigations. This was a preponderant condition for the big expansion experienced by the Brazilian fish cytogenetics.

The "Final Remarks" highlights some key contributions to fish evolutionary cytogenetics from MSc and PhD theses produced, as well as from other results that were led by our research team, some of them with significant colaborations of other national and international research groups.

Laboratory site at the Universidade Federal de São Carlos: (http://www.lcp.ufscar.br)

Abbreviations used

UFSCar	Universidade Federal de São Carlos
INPA	Instituto Nacional de Pesquisas da Amazônia
USP	Universidade de São Paulo
UFRJ	Universidade Federal do Rio de Janeiro
PPGGEv	Programa de Pós-Graduação em Genética Evolutiva e Biologia Molecular
BADPI	Programa de Pós-Graduação em Biologia de Água Doce e Pesca Interior
PPGERN	Programa de Pós-Graduação em Ecologia e Recursos Naturais
PPGCB – Gene	Programa de Pós-Graduação em Ciências Biológicas – Genética
PPGCB – Ecol	Programa de Pós-Graduação em Ciências Biológicas – Ecologia
CAPES	Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico

Catalogue of MSc Dissertations and PhD Theses

Note: Titles of Theses and Dissertations maintain the taxonomic and/or systematic data as they were originally employed. The classification of some species and genera were later updated by review studies (Reis et al. 2003; Oliveira et al. 2011; FishBase), according to the section: **taxon/taxa analyzed**.

I. ORDER CHARACIFORMES

I.1. Family Characidae

I.1.1. Genus *Astyanax* Baird & Girard, 1854

I.1.1.1. MSc Dissertation by Sandra Morelli (1981): Aspectos citogenéticos do gênero *Astyanax* (Pisces, Characidae) / Cytogenetic studies in the genus *Astyanax*

(Pisces, Characidae). **Taxa analyzed:** *A. fasciatus* (Cuvier, 1819), *A. bimaculatus* (Linnaeus, 1758), *A. schubarti* Britski, 1964, *A. scabripinnis* (Jenyns, 1842) – UFSCar / PPGERN / CNPq

I.1.1.2. PhD Thesis by Orlando Moreira Filho (1989): A diversidade no complexo *scabripinnis* (Pisces, Characidae, Tetragonopterinae). Análises citogenéticas e morfológicas. / Diversity investigation in the *scabripinnis* complex (Pisces, Characidae, Tetragonopterinae). Cytogenetic and morphological analyses. **Taxon analyzed:** *A. scabripinnis* – UFSCar / PPGERN

I.1.1.3. MSc Dissertation by Heloisa Helena Paganelli (1990): A variabilidade cromossômica no gênero *Astyanax* (Pisces, Characidae) e seu significado para a sistemática e evolução do grupo / Chromosomal variability in the genus *Astyanax* (Pisces, Characidae) and its significance for the systematics and evolution of the group. **Taxa analyzed:** *A. bimaculatus, A. fasciatus, A. schubarti, A. taeniatus* (Jenyns, 1842) -UFSCar / PPGERN

I.1.1.4. MSc Dissertation by Álvaro José Justi (1993): Caracterização cariotípica de populações de *Astyanax fasciatus* (Characidae) de bacias hidrográficas distintas / Karyotype characterization of *Astyanax fasciatus* populations (Characidae) from different river basins. **Taxon analyzed:** *A. fasciatus* – UFSCar / PPGGEv / FAPESP

I.1.1.5. MSc Dissertation by Vera Elisa Vicente (1994): Estudo do cromossomo B em três populações de *Astyanax scabripinnis* (Characidae) / B chromosome analysis in three *Astyanax scabripinnis* populations (Characidae). **Taxon analyzed:** *A. scabripinnis* – UFSCar / PPGGEv / FAPESP

I.1.1.6. MSc Dissertation by Issakar Lima Souza (1996): Estudos citogenéticos em populações de *Astyanax scabripinnis* (Characidae) pertencentes a dois riachos de diferentes bacias do Sudeste Brasileiro / Cytogenetic studies in populations of *Astyanax scabripinnis* (Characidae) from two different river basins of Southeastern Brazil.**Taxon analyzed:** *A. scabripinnis* – UFSCar / PPGGEv / FAPESP

I.1.1.7. PhD Thesis by Carlos Alberto Mestriner (1997): Caracterização molecular e citológica do DNA repetitivo de *Astyanax scabripinnis* (Pisces, Characidae) portador de cromossomos supranumerários / Molecular and cytological characterization of repetitive DNAs in *Astyanax scabripinnis* (Pisces, Characidae) carrying supernumerary chromosomes. **Taxon analyzed:** *A. scabripinnis* – UFSCar / PPGGEv / FAPESP

I.1.1.8. MSc Dissertation by María Pía Heras (1998): Estudos citogenéticos em *Astyanax fasciatus* (Characidae) de alguns rios do Brasil / Cytogenetic studies in *Astyanax fasciatus* (Characidae) from some Brazilian rivers. **Taxon analyzed:** *A. fasciatus* populations – UFSCar / PPGGEv / CNPq

I.1.1.9. MSc Dissertation by Daniela Morilha Néo (1999): Distribuição dos cromossomos B presentes em *Astyanax scabripinnis* (Characidae) ao longo do Ribeirão Grande na região de Campos do Jordão–SP / B chromosomes distribution in *Astyanax scabripinnis* (Characidae) along the Grande Stream, Campos do Jordão region–SP. **Taxon analyzed:** *A. scabripinnis* – UFSCar / PPGGEv / FAPESP

I.1.1.10. PhD Thesis by Dagmar Aparecida de Marco Ferro (2000): Análises cariotípicas dos cromossomos B em populações de *Astyanax scabripinnis* (Pisces, Characidae) / Karyotypic analyses of B chromosomes in *Astyanax scabripinnis* populations (Pisces, Characidae). **Taxon analyzed:** *A. scabripinnis* – UFSCar / PPGGEv / CNPq

I.1.1.11. MSc Dissertation by Monique Mantovani (2001): Citogenética comparativa entre populações de *Astyanax scabripinnis* (Characidae) da bacia do rio Paranapanema / Comparative cytogenetics among populations of *Astyanax scabripinnis* (Characidae) from the Paranapanema River basin. **Taxon analyzed:** *A. scabripinnis* – UFSCar / PPGGEv / FAPESP

I.1.1.12. MSc Dissertation by Luciano Douglas dos Santos Abel (2001): A variabilidade do complexo de espécies *scabripinnis* (Characidae) como estratégia adaptativa. Estudo da diversidade cariotípica do grupo com ênfase em populações da bacia do rio São Francisco / The variability of the *scabripinnis* species complex (Characidae) as an adaptive strategy. Analysis of the karyotypic diversity emphasizing populations from the São Francisco River basin. **Taxon analyzed:** *A. scabripinnis* – UFSCar / PPGGEv / FAPESP

I.1.1.13. PhD Thesis by Rubens Pazza (2005): Contribuição citogenética à análise da biodiversidade em *Astyanax fasciatus* (Pisces, Characidae) / Cytogenetic contribution to biodiversity analysis in *Astyanax fasciatus* (Pisces, Characidae). **Taxon analyzed:** *A. fasciatus* – UFSCar / PPGGEv / FAPESP

I.1.1.14. PhD Thesis by Wellington Adriano Moreira (2009): Análise citogenética de espécies de *Astyanax* (Characiformes) na região de transposição do rio Piumhi / Cytogenetic analysis of *Astyanax* species (Characiformes) from the transposition region of the Piumhi River. **Taxa analyzed:** *A. scabripinnis*, *A. bimaculatus*, *A. lacustris* (Lütken, 1875), *A. altiparanae* Garutti & Britski, 2000 – UFSCar / PPGGEv / FAPESP

I.1.2. Genus *Bryconamericus* Eigenmann, 1907

I.1.2.1. MSc Dissertation by Adriane Pinto Wasko (1996): Estudos citogenéticos no gênero *Bryconamericus* (Pisces, Characidae). Uma abordagem citotaxonômica-evolutiva / Cytogenetic studies in the *Bryconamericus* genus (Pisces, Characidae). A cytotaxonomic-evolutionary approach. **Taxa analyzed:** *Bryconamericus* sp A-E, *Piabina argentea* Reinhardt, 1867 – UFSCar / PPGGEv / CNPq

I.1.3. Genus *Moenkhausia* Eigenmann, 1903

I.1.3.1. MSc Dissertation by Elisangela Santana de Oliveira Dantas (2002): Estudos citogenéticos entre três espécies de *Moenkhausia* (Characidae, Tetragonopterinae) de localidades diferentes / Cytogenetic studies among three *Moenkhausia* species (Characidae, Tetragonopterinae), from different localities **Taxa analyzed:** *Moenkhausia sanctae filomenae* (Steindachner,1907), *M. intermedia* Eigenmann, 1908, *Moenkhausia* sp. – UFSCar / PPGGEv / FAPESP

I.1.4. Subfamily Bryconinae

I.1.4.1. Genus *Brycon* Müller & Troschel, 1844

I.1.4.1.1. MSc Dissertation by Vladimir Pavan Margarido (1995): Uma contribuição à citogenética de Bryconinae (Characiformes, Characidae) / A contribution to Bryconinae cytogenetics (Characiformes, Characidae). **Taxa analyzed**: *Brycon brevicauda* Günther, 1864, *B. lundi* Lütken, 1875, *B. orbignyanus* (Valenciennes,1850), *B. microlepis* Perugia, 1897), *B. cephalus* (Günther, 1869), *B. insignis* Steindachner, 1877, *Brycon sp*.- UFSCar / PPGGEv / CAPES

I.1.4.1.2. PhD Thesis by Adriane Pinto Wasko (2000): Marcadores cromossômicos e moleculares no gênero *Brycon* (Characidae): uma contribuição à biologia evolutiva e à conservação biológica destes peixes / Chromosomal and molecular markers in the genus *Brycon* (Characidae): a contribution to its evolutionary and conservation biology. **Taxa analyzed**: *Brycon lundii*, *B. orbignyanus*, *B. microlepis*, *B. cephalus, B. brevicauda, B. insignis, Brycon* sp. – UFSCar / PPGGEv / FAPESP/CNPq

I.1.5. Miscelaneous groups in Characidae

I.1.5.1. MSc Dissertation by Ana Luiza de Brito Silva Portela (1987): Citogenética de peixes da subfamília Tetragonopterinae (Characidae) / Fish cytogenetics of the Tetragonopterinae subfamily (Characidae). **Taxa analyzed**: *Tetragonopterus chalceus* Spix et Agassiz, 1829, *Piabina argentea, Bryconamericus stramineus* Eigenmann, 1908*, Moenkhausia costae* (Steindachner,1907), *M. intermedia* Eigenmann, 1908*, Deuterodon pedri* Eigenmann, 1908 – USP / PPGCB – Gene / CAPES

I.1.5.2. MSc Dissertation by Sandra Cristina Pfister (1997): Contribuição aos estudos cariotípicos da família Characidae da bacia do rio São Francisco – Três Marias (MG) / A contribution to the karyotypic studies in the family Characidae from the São Francisco River basin – Três Marias (MG).**Taxa analyzed**: *Roeboides xenodon* (Reinhardt, 1851), *Orthospinus franciscensis* (Eigenmann, 1914), *Bryconops affinis* (Günther, 1864), *Hemigrammus marginatus* Ellis, 1911, *Moenkhausia costae* – UFSCar / PPGGEv / CNPq

I.1.5.3. PhD Thesis by Issakar Lima Souza (2003): rDNAs nucleares e bandamentos cromossômicos em Salmininae e *Astyanax scabripinnis* (Characidae) / Nuclear rDNAs and chromosome banding in Salmininae and *Astyanax scabripinnis* (Characidae). **Taxa analyzed**: *A.scabripinnis***,** *Salminus brasiliensis* (Cuvier, 1816)*, S. hilarii* Valenciennes, 1850 – UFSCar / PPGGEv / CNPq / FAPESP

I.1.5.4. MSc Dissertation by Wellington Adriano Moreira Peres (2005): Análise da diversidade cariotípica de Characidae da bacia do São Francisco / Analysis on karyotypic diversity of Characidae fishes from the São Francisco River basin. **Taxa analyzed**: *Orthospinus franciscensis*, *Serrapinnus heterodon* (Eigenmann, 1915)*, S. piaba* (Lütken,1875), *Astyanax fasciatus*, *A. bimaculatus*, *Haseamania nana* (Lütken, 1875), *Piabina argentea* – UFSCar / PPGGEv / FAPESP

I.2. Family Prochilodontidae

I.2.1. Genus *Prochilodus* Agassiz, in Spix et Agassiz, 1829

I.2.1.1. MSc Dissertation by Erica Pauls (1981): Evidências de um sistema de cromossomos supranumerários em *Prochilodus scrofa* Steindachner, 1881 (Pisces, Prochilodontidae) / Evidences for a supernumerary chromosome system in *Prochilodus scrofa* Steindachner, 1881 (Pisces, Prochilodontidae). **Taxon analyzed**: *Prochilodus lineatus* (Valenciennes, 1836), cited as *P. scrofa* – UFSCar / PPGERN / CNPq

I.2.1.2. PhD Thesis by Erica Pauls (1985): Considerações sobre evolução cromossômica e sistema de cromossomos supranumerários em espécies do gênero *Prochilodus* (Pisces, Prochilodontidae) / Considerations on chromosomal evolution and supernumerary chromosome systems in *Prochilodus* species (Pisces, Prochilodontidae). **Taxa analyzed**: *P. lineatus* (cited as *P. scrofa*), *P. vimboides* Kner, 1859*, P. brevis* Steindacher, 1875 (cited as *P. cearensis* Steindachner, 1911), *P. argenteus* Agassiz, 1829, *P. margravii* (rejected by ICZN; under synonymy of *P. argenteus*), *P. costatus* Valenciennes, 1850 (cited as *P. affinis* Lütken, 1875, *P. nigricans* Agassiz, 1829 – UFSCar / PPGERN / CNPq

I.2.1.3. MSc Dissertation by Zélia Isabel Cavallaro (1992): Estudos comparativos sobre os cromossomos B de *Prochilodus scrofa* Steindachner, 1881 (Pisces, Prochilodontidae) / Comparative studies on B chromosomes of *Prochilodus scrofa* Steindachner, 1881 (Pisces, Prochilodontidae). **Taxon analyzed**: *P. lineatus*, cited as *P. scrofa* – UFSCar / PPGERN / CAPES

I.2.1.4. PhD Thesis by Terumi Hatanaka (2000): Marcadores cromossômicos e moleculares no peixe *Prochilodus marggravii*: uma espécie de interesse econômico no rio São Francisco / Chromosomal and molecular markers in *Prochilodus marggravii*, a fish species with economic significance from the São Francisco River. **Taxon analyzed**: *P. argenteus* (cited as *P. margravii*: rejected by ICZN)- UFSCar / PPGGEv / FAPESP

I.2.1.5. PhD Thesis by Célia Maria de Jesus (2001): Caracterização de sequências repetitivas no genoma de *Prochilodus lineatus* (Prochilodontidae) portador de cromossomos B / Characterization of repetitive sequences in the genome of *Prochilodus lineatus* (Prochilodontidae) carrying B chromosomes. **Taxon analyzed**: *P. lineatus* – UFSCar / PPGGEv / CNPq / FAPESP

I.3. Family Parodontidae

I.3.1. MSc Dissertation by Orlando Moreira Filho (1983): Estudos na família Parodontidae (Pisces, Characiformes – cited as Cypriniformes) da bacia do rio Passa-Cinco (SP): aspectos citogenéticos e considerações correlatas / Studies in Parodontidae species (Pisces, Characiformes) from the Passa-Cinco River Basin (SP): cytogenetic and correlated considerations.**Taxa analyzed**: *Apareiodon affinis* (Steindachner, 1879), *A. ibitiensis* Campos, 1944*, A. piracicabae* (Eigenmann, 1907), *Parodon nasus* Kner, 1859 (cited as *P. tortuosus* Eigenmann & Norris, 1900) – UFSCar / PPGERN

I.3.2. MSc Dissertation by Célia Maria de Jesus (1996): Contribuição aos estudos citogenéticos na família Parodontidae (Characiformes) / Contribution to cytogenetic studies in the family Parodontidae (Characiformes). **Taxa analyzed**: *Parodon nasus* (cited as *P. tortuosus*), *P. pongonensis* (Allen, 1942) (cited as *Parodon* sp), *Apareiodon affinis, A. ibitiensis, A. piracicabae, A.vitattus* Garavello, 1977- UFSCar / PPGGEv / FAPESP

I.3.3. PhD Thesis by Vera Elisa Vicente (2001): Estudos citogenéticos e moleculares em *Parodon hilarii* e correlações com outras espécies da família Parodontidae (Characiformes) / Cytogenetic and molecular studies in *Parodon hilarii* and correlations with other Parodontidae species. **Taxa analyzed**: *P. hilarii* Reinhardt, 1866, *P. nasus* (cited as *P. tortuosus*) – UFSCar / PPGGEv / CNPq

I.3.4. MSc Dissertation by Elisangela Bellafronte da Silva (2004): Estudos citogenéticos comparativos em espécies do gênero *Parodon* (Parodontidae) / Comparative cytogenetics in *Parodon* species (Parodontidae).**Taxon analyzed**: *P. nasus* – UFSCar / PPGGEv / CNPq

I.3.5. PhD Thesis by Josiane Baccarin Traldi (2015): Investigação do papel dos DNAs repetitivos na evolução cromossômica de espécies de *Apareiodon* (Characiformes, Parodontidae) / Investigation on the role of repetitive DNAs in the chromosomal evolution of *Apareiodon* species (Characiformes, Parodontidae). **Taxa analyzed**: *A. cavalcante* Pavanelli & Britski, 2003, *A. machrisi* Travassos, 1957, *A. argenteus* Pavanelli & Britski, 2003, *A. davisi* Fowler, 1941, *Apareiodon* sp. 1, *Apareiodon* sp. 2 – UFSCar / PPGGEv / FAPESP

I.4. Family Erythrinidae

I.4.1. MSc Dissertation by Lucia Giuliano Caetano (1986): Estudo citogenético em *Hoplerythrinus unitaeniatus* (Pisces, Erythrinidae) de diferentes bacias hidrográficas brasileiras / Cytogenetic studies in *Hoplerythrinus unitaeniatus* (Pisces, Erythrinidae) from different Brazilian river basins. **Taxon analyzed**: *H. unitaeniatus* (Agassiz, 1829) – UFSCar / PPGERN / CNPq

I.4.2. MSc Dissertation by Jorge Abdala Dergam dos Santos (1989): O cariótipo de *Hoplias malabaricus* em populações da bacia do São Francisco e do Alto Paraná. Considerações citotaxonômicas / The karyotype of *Hoplias malabaricus* populations from the São Francisco and High Paraná River basins. Cytotaxomic considerations. **Taxon analyzed**: *H. malabaricus* (Block, 1794) karyomorphs D, F – USP / PPGCB – Gene / CAPES

I.4.3. PhD Thesis by Sandra Morelli (1998): Citogenética evolutiva em espécies do gênero *Hoplias*, grupo *H. lacerdae*. Macroestrutura cariotípica, heterocromatina constitutiva e regiões organizadoras de nucléolos / Evolutionary cytogenetics in *Hoplias lacerdae* species group. Karyotype macrostructure, constitutive heterochromatin and nucleolus organizing regions. **Taxon analyzed**: *H. lacerdae* Miranda Ribeiro, 1908 species group – UFSCar / PPGGEv / CNPq

I.4.4. PhD Thesis by Guassenir Gonçalves Born (2000): Estudo da diversidade cariotípica no grupo *Hoplias malabaricus* (Pisces, Erythrinidae). Cariótipo 2n=42

/ Study on the karyotypic diversity in the *Hoplias malabaricus* species group (Pisces, Erythrinidae). The karyotype 2n=42. **Taxon analyzed**: *H. malabaricus* karyomorphs A, B – UFSCar / PPGGEv / CAPES

I.4.5. MSc Dissertation by Débora Diniz Bezerra (2002): Estudos citogenéticos populacionais em *Hoplerythrinus unitaeniatus* (Pisces, Erythrinidae). Análise da biodiversidade / Population cytogenetic studies in *Hoplerythrinus unitaeniatus* (Pisces, Erythrinidae). Biodiversity analysis. **Taxon analyzed**: *H. unitaeniatus* – UFSCar / PPGGEv / CNPq

I.4.6. MSc Dissertation by Marcelo Ricardo Vicari (2003): Citogenética comparativa de *Hoplias malabaricus* (Pisces, Erythrinidae). Estudos em região divisora de águas das bacias dos rios Tibagi, Iguaçu, Ivaí e Ribeira (Ponta Grossa, PR) / Comparative cytogenetics of *Hoplias malabaricus* (Pisces, Erythrinidae). Studies in the water divisor region of the Tibagi, Iguaçu, Ivaí and Ribeira Rivers (Ponta Grossa, PR). **Taxon analyzed**: *H. malabaricus* – UFSCar / PPGGEv / FAPESP

I.4.7. MSc Dissertation by Marcelo de Bello Cioffi (2010): Marcadores cromossômicos em *Hoplias malabaricus* (Characiformes, Erythrinidae). Citogenética comparativa entre cariomorfos / Chromosome markers in *Hoplias malabaricus* (Characiformes, Erythrinidae. Comparative cytogenetics among karyomorphs. **Taxon analyzed**: *H. malabaricus* karyomorphs A, B, C, D – UFSCar / PPGGEv / FAPESP

I.4.8. MSc Dissertation by Daniel Rodrigues Blanco (2010): Caracterização citogenética em populações alopátricas do gênero *Hoplias*, com enfoque nos grupos *malabaricus* e *lacerdae* / Cytogenetic characterization of allopatric populations of the *Hoplias* genus, focusing on the *malabaricus* and *lacerdae* groups. **Taxa analyzed**: *H. malabaricus*, *H. aimara* (Valenciennes, 1847), *H. intermedius* (Günther, 1864) – UFSCar / PPGGEv / FAPESP

I.4.9. PhD Thesis by Marcelo de Bello Cioffi (2011): Evolução cromossômica na família Erythrinidae. Mapeamento citogenético de DNAs repetitivos e microdissecção de cromossomos sexuais / Chromosome evolution in the Erythrinidae family. Cytogenetic mapping of repetitive DNAs and microdissection of sex chromosomes. **Taxa analyzed**: *Hoplias malabaricus* karyomorphs A, B, C, D, *Erythrinus erythrinus* (Bloch & Schneider, 1801) karyomorphs A, D – UFSCar / PPGGEv / FAPESP

I.4.10. MSc Dissertation by Nícolas Fernandes Martins (2013): Diferenciação cromossômica em *Erythrinus erythrinus* (Characiformes, Erythrinidae) / Chromosomal differentiation in *Erythrinus erythrinus* (Characiformes, Erythrinidae). **Taxon analyzed**: *E. erythrinus* karyomorphs A, C – UFSCar / PPGGEv / CAPES

I.4.11. MSc Dissertation by Juliana de Fátima Martinez (2014): *Hoplerythrinus unitaeniatus* (Characiformes, Erythrinidae): um complexo de espécies. Estudos citogenéticos e moleculares / *Hoplerythrinus unitaeniatus* (Characiformes, Erythrinidae): a species complex. Cytogenetic and molecular analyses. **Taxon analyzed**: *H. unitaeniatus* – UFSCar / PPGGEv / FAPESP

I.4.12. MSc Dissertation by Ezequiel Aguiar de Oliveira (2015): Evolução cromossômica em peixes da família Erythrinidae (Characiformes). Citogenética compa-

rativa entre espécies do gênero *Hoplias* / Chromosome evolution in the fish family Erythrinidae (Characiformes). Comparative cytogenetics among *Hoplias* species. **Taxa analyzed**: *H. aimara*, *H. brasiliensis* (Agassiz, 1829), *H. lacerdae*, *H. intermedius* – UFSCar / PPGGEv / CAPES

I.5. Family Serrasalmidae (Former Serrasalminae, Characidae)

I.5.1. MSc Dissertation by Marta Margarete Cestari (1990): Diferenciação cromossômica no gênero *Serrasalmus* La Cèpede, 1803 e evolução do cariótipo em Serrasalminae (Pisces, Characidae) / Chromosomal differentiation in the genus *Serrasalmus* La Cèpede, 1803 and karyotypic evolution in Serrasalminae (Pisces, Characidae). **Taxa analyzed**: *S. spilopleura* Kner, 1858, *S. humerallis* Valenciennes, 1850, *S. brandti* (Lütken, 1875) – UFSCar / PPGERN / CNPq

I.5.2. PhD Thesis by Marta Margarete Cestari (1996): Estudos citogenéticos e genético-bioquímicos do gênero *Serrasalmus* (Pisces, Serrasalminae) / Cytogenetic and genetic-biochemical studies in the genus *Serrasalmus* (Pisces, Serrasalminae).**Taxa analyzed**: *S. spilopleura*, *S. marginatus* Valenciennes, 1837 -UFSCar / PPGGEv / CAPES

I.5.3. PhD Thesis by Jorge Ivan Rebelo Porto (1999): Análises cariotípicas e sequenciamento de DNA mitocondrial em populações de *Mylesinus paraschomburgkii* (Characiformes, Serrasalminae) da bacia amazônica / Karyotypic analyses and mtDNA sequencing in *Mylesinus paraschomburgkii* populations (Characiformes, Serrasalminae) from the Amazon Basin. **Taxon analyzed**: *M. paraschomburgkii* Jégu, Santos & Ferreira, 1989 – INPA/BADPI/CNPq

I.5.4. PhD Thesis by Celeste Mutuko Nakayama (2007): Citogenética molecular comparativa do DNAr 18S e DNAr 5S em piranhas (Characidae, Serrasalminae) da Amazônia Central / Comparative molecular cytogenetics of the 18S and 5S rDNAs in piranhas (Characidae, Serrasalminae) from the Central Amazon. **Taxa analyzed**: *Serrasalmus altispinnis* Merchx, Jégu & Santos, 2000, *S. elongatus* Kner, 1858, *S. gouldingi* Fink & Machado-Allison, 1992, *S. rhombeus* (Linnaeus, 1766), *S. serrulatus* (Valenciennes, 1850), *S. maculatus* Kner, 1858, *S.* cf. *rhombeus*, *Pygocentrus nattereri* Kner, 1858, *Pristobrycon striolatus* (Steindachner, 1908), *Catoprion mento* (Cuvier, 1819) – UFSCar / PPGGEv / CNPq

I.6. Family Triportheidae (former Triportheinae, Characidae)

I.6.1. PhD Thesis by José das Neves Falcão (1988): Caracterização cariotípica em peixes do gênero *Triportheus* (Teleostei, Characiformes, Characidae) / Karyotypic characterization of *Triportheus* fish (Teleostei, Characiformes, Characidae). **Taxa analyzed**: *T. signatus* (Garman, 1890), *T. angulatus* (Spix & Agassiz, 1829) (cited as *T. flavus* Cope, 1872), *T. albus* Cope, 1872, *T. culter* (Cope, 1872), *T. auritus* (Valenciennes, 1850) (cited as *T. elongatus* (Günther, 1864) – USP / PPGCB – Gene / CAPES

I.6.2. PhD Thesis by Roberto Ferreira Artoni (1999): Citogenética do sistema de cromossomos sexuais ZZ/ZW no gênero *Triportheus* (Pisces, Characidae) / Cy-

togenetics of the ZZ/ZW sex chromosome system in the genus *Triportheus* (Pisces, Characidae). **Taxa analyzed**: *Triportheus* cf. *auritus*, cited as *T*. cf. *elongatus*, *T. guentheri* (Garman, 1890), *T. nematurus* (Kner, 1858) (cited as *T. paranensis* (Günther, 1874) – UFSCar / PPGGEv / FAPESP

I.6.3. PhD Thesis by Débora Diniz Bezerra (2007): Origem e diferenciação do sistema de cromossomos sexuais ZZ/ZW em *Triportheus* (Characiformes, Characidae). Citogenética, mapeamento de genes ribossomais e microdissecção cromossômica / Origin and differentiation of the ZZ/ZW sex chromosome system in *Triportheus* (Characiformes, Characidae). Cytogenetic mapping of ribosomal genes and chromosomal microdissection. **Taxa analyzed**: *T. nematurus*, *T. guentheri*, *T. trifurcatus* (Castelnau, 1855), *T. auritus*, *T. angulatus*, *T. albus*, *Triportheus cf. signatus* – UFSCar / PPGGEv / CNPq

I.6.4. PhD Thesis by Cássia Fernanda Yano (2016): Estudos evolutivos no gênero *Triportheus* (Characiformes, Triportheidae) com enfoque na diferenciação do sistema de cromossomos sexuais ZZ/ZW / Evolutionary studies in the *Triportheus* genus (Characiformes, Triportheidae) foccusing on the differentiation of the ZZ/ZW sex chromosome system. **Taxa analyzed**: *T. auritus*, *T. guentheri*, *T. albus*, *Triportheus aff. rotundatus* (Jardine, 1841), *T. nematurus*, *T. signatus*, *T. trifurcatus*, *T. pantanensis* Malabarba, 2004 – UFSCar / PPGGEv / CAPES

I.7. Family Curimatidae

I.7.1. PhD Thesis by Eliana Feldberg (1990): Estudos citogenéticos em doze espécies de peixes da família Curimatidae (Characiformes) da Amazônia Central / Cytogenetic studies of twelve Curimatidae species (Characiformes) from the Central Amazon. **Taxa analyzed**: *Potamorhina pristigaster* (Steindachner, 1876), *P. altamazonica* (Cope, 1878), *P. latior* (Spix & Agassiz, 1829), *Curimata ocellata* (Eigenmann & Eigenmann, 1889), *C. vittata* (Kner, 1858), *C. kneri* (Steindachner, 1876), *C. cyprinoides* (Linnaeus, 1766), *Curimata* sp, *Psectrogaster rutiloides* (Kner, 1858), *Curimatella alburna* (Müller & Troschel, 1844), *C. meyeri* (Steindachner, 1882) – INPA / BADPI

I.7.2. MSc Dissertation by Paulo Cesar Venere (1991): Citogenética comparativa de peixes da família Curimatidae (Characiformes) / Comparative cytogenetics of Curimatidae fish (Characiformes). **Taxa analyzed**: *Cyphocharax gilberti* (Quoy et Gaimard, 1824), *C. modestus* (Fernández-Yépez, 1948), *C. nagellii* (Steindachner, 1881), *C. vanderi* (Britski, 1980), *C. voga* (Hensel, 1870), *Cyphocharax* sp., *Steindachnerina elegans* (Steindachner, 1874), *Steindachnerina* sp., *S. insculpta* (Fernández-Yépez, 1948), *Curimatella lepidura* (Eigenmann & Eigenmann, 1889) – UFSCar / PPGERN / CAPES

I.7.3. PhD Thesis by Rosângela Martins de Oliveira (2011): Citogenética clássica e molecular de três espécies de curimatídeos, com ênfase no cromossomo B de *Cyphocharax nagelli* (Characiformes, Curimatidae) / Conventional and molecular cytogenetics in three curimatid species, with emphasis on the B chromosome of *Cyphocharax nagelli* (Characiformes, Curimatidae). **Taxa analyzed**: *C. nagelli*, *C. modestus*, *Steindachnerina insculpta* – UFSCar / PPGGEv / CAPES/CNPq

I.8. Family Crenuchidae (former Characidiinae, Characidae)

I.8.1. MSc Dissertation by Carlos Suetoshi Miyazawa (1991): Estudos citogenéticos em peixes do grupo *Characidium* (Characidiinae, Characidae), de distintas bacias hidrográficas / Cytogenetic studies in *Characidium* (Characidiinae, Characidae) species from different hydrographic basins.**Taxa analyzed**: *C. pterostictum* Gomes, 1947, *Characidium* cf. *zebra* Eigenmann, 1909*, Characidium* cf. *lagosantense* Travassos, 1947, *Characidium* sp. – UFSCar / PPGERN / CAPES

I.9. Family Anostomidae

I.9.1. MSc Dissertation by Carlos Alberto Mestriner (1993): Análise das regiões organizadoras de nucléolo e investigação do sistema XX/XY descrito para *Leporinus lacustris* (Pisces, Anostomidae) / Analyses of the nucleolus organizer regions and investigation of the XX/XY sex system of *Leporinus lacustris* (Pisces, Anostomidae). **Taxon analyzed**: *L. lacustris* Campos, 1945 – UFSCar / PPGGEv / FAPESP

I.9.2. MSc Dissertation by Wagner Franco Molina (1995): Cromossomos sexuais e polimorfismo cromossômico no gênero *Leporinus* (Pisces, Anostomidae) / Sex chromosomes and chromosomal polymorphism in the genus *Leporinus* (Pisces, Anostomidae).**Taxa analyzed**: *L. elongatus* Valenciennes, 1850, *L. obtusidens* (Valenciennes, 1836), *L. reinhardti* Lütken, 1875, *Leporinus* aff. *elongatus* – UFSCar / PPGGEv / CAPES

I.9.3. MSc Dissertation by Cesar Martins (1997): Novas contribuições à citogenética de Anostomidae (Pisces, Characiformes). Citotaxonomia e filogenia no gênero *Schizodon* / New contributions to cytogenetics of Anostomidae (Pisces, Characiformes). Cytotaxonomy and phylogeny of the genus *Schizodon*. **Taxa analyzed**: *S. altoparanae* Garavello & Britski, 1990, *S. nasutus* Kner, 1858, *S. knerii* (Steindachner, 1875), *S. vittatus* (Valenciennes, 1850), *S. fasciatus* Spix & Agassiz, 1829, *S. borelli* (Boulenger, 1900), *S. isognathus* Kner, 1858, *S. intermedius* Garavello & Britski, 1990 – UFSCar / PPGGEv / CNPq

I.9.4. MSc Dissertation by Suelen Regina Lopes Krichanã (1999): Contribuição ao estudo citogenético da família Anostomidae (Pisces, Characiformes) na região Amazônica / Contribution to the cytogenetics of the family Anostomidae (Pisces, Characiformes) from the Amazon region- **Taxa analyzed**: *Laemolita taeniata* (Kner, 1858), *Leporinus agassizii* Steindachner, 1876, *Leporinus cylindriformis* Borodin, 1929, *Leporinus fasciatus*, *Leporinus friderici*, *Leporinus granti* Eigenmann, 1912, *Rhythiodus microlepis*, *Schizodon fasciatus* – UFSCar / PPGGEv

I.9.5. PhD Thesis by Cesar Martins (2000): Organização do DNA ribossômico 5S no genoma de peixes, com ênfase em *Leporinus* / Organization of the 5S rDNA in the fish genome, with emphasis on *Leporinus*. **Taxa analyzed**: *L. elongatus, L. obtusidens*, *L. friderici* (Block, 1794), *L. cf. elongatus*, *L. reinhardti*, *L. piau* Fowler, 1941, *L. desmotes* Fowler, 1914, *L. conirostris* Steindachner, 1875, *Schizodon altoparanae*, *S. borelli*, *S. isognathus*, *S. nasutus*, *S. knerii*, *S. vittatus* – UFSCar / PPGGEv / FAPESP

I.9.6. PhD Thesis by Vladimir Pavan Margarido (2000): Uma contribuição à citogenética de Anostomidae, com ênfase na variabilidade das regiões organizadoras de nucléolos no gênero *Leporinus* (Pisces, Characiformes) / Contribution to Anostomidae cytogenetics, with emphasis on the variabilty of the nucleolar organizing regions in the genus *Leporinus* (Pisces, Characiformes). **Taxa analyzed**: *L. copelandii* Steindachner, 1875, *L. conirostris*, *L. desmotes*, *L. elongatus*, *L.* cf. *elongatus*, *L. fasciatus* (Block, 1794), *L. friderici*, *L. lacustris*, *L. macrocephalus* Garavello and Britski, 1988, *L. mormyrops* Steindachner, 1875, *L. obtusidens*, *L. octofasciatus* Steindacher, 1915, *L. piau*, *L. reinhardti*, *L. striatus Kner*, 1858, *L. taeniatus* Lütken, 1875, *L. tigrinus* Borodin, 1929 – UFSCar / PPGGEv / CNPq

I.9.7. PhD Thesis by Cecília Teixeira de Aguilar (2001): Estudos citogenéticos e moleculares em populações brasileiras de *Leporellus vittatus* (Characiformes, Anostomidae) / Cytogenetic and molecular studies in Brazilian populations of *Leporellus vittatus* (Characiformes, Anostomidae). **Taxon analyzed**: *L. vittatus* (Valenciennes, 1850) – UFSCar / PPGGEv / CAPES

II. ORDER SILURIFORMES

II.1. Family Heptapteridae

II.1.1. MSc Dissertation by Alberto Sergio Fenocchio (1987): Polimorfismo cromossômico em *Rhamdia hilarii* (Pisces, Heptapteridae citado como Pimelodidae) / Chromosomal polymorphism in *Rhamdia hilarii* (Pisces, Heptapteridae). **Taxon analyzed**: *R. quelen* (Quoy & Gaimard, 1824) (cited as *Rhamdia hilarii* (Valenciennes, 1840) – USP / PPGCB – Gene / CNPq

II.2. Family Loricariidae

II.2.1. MSc Dissertation by Roberto Ferreira Artoni (1996): Estudos citogenéticos na família Loricariidae, com especial ênfase no gênero *Hypostomus* Lacepede (1803) – Pisces, Siluriformes / Cytogenetic studies in the family Loricariidae, with special emphasis on the *Hypostomus* genus Lacepede (1803) – Pisces, Siluriformes. **Taxa analyzed**: *H. ancistroides* (Ihering, 1911), *H. regani* (Ihering, 1905), *H. albopunctatus* (Regan, 1908), *Hypostomus* aff. *auroguttatus* Kner, 1854, *Squaliforma emarginata* (Valenciennes, 1840) (cited as *Hypostomus emarginatus* Valenciennes in Cuvier et Valenciennes, 1840), *Hypostomus* sp., *Rhinelepsis aspera* Spix & Agassiz, 1829, *Liposarcus* sp., *Pogonopoma wertheimeri* (Steindachner, 1867), *Panaque* cf. *nigrolineatus* (Peters, 1877), *Hemiancistrus* sp., *Sturisoma* cf. *nigrirostrum* Fowler, 1940 -UFSCar / PPGGEv / CNPq

II.2.2. PhD Thesis by Lúcia Giuliano-Caetano (1998): Polimorfismo cromossômico Robertsoniano em populações de *Rineloricaria latirostris* (Pisces, Loricariidae) / Robertsonian chromosomal polimorphism in *Rineloricaria latirostris* populations (Pisces, Loricariidae).**Taxa analyzed**: *R. rialatirostris* (Boulenger, 1900), *R. pentamaculata* Langeani & Araujo, 1994 -UFSCar / PPGGEv / CAPES

II.2.3. MSc Dissertation by Fábio Mendes Camilo (2004): Estudos citogenéticos de algumas espécies de peixes da família Loricariidae pertencentes à bacia do rio Piracicaba / Cytogenetic studies in Loricariidae fish species from the Piracicaba River basin.**Taxa analyzed**: *Corumbatai acuestae* Britsky, 1997, *Liposarcus anisitsi* (Eigenmann & Kennedy, 1903), *Hypostomus albopunctatus* -UFSCar / PPGGEv

II.2.4. PhD Thesis by Sandra Mariotto (2008): Estudo citogenético clássico e molecular em quinze espécies da tribo Ancistrini (Siluriformes, Loricariidae) de três bacias hidrográficas brasileiras / Conventional and molecular cytogenetic studies in 15 Ancistrini species (Siluriformes, Loricariidae) from three Brazilian hidrographic basins. **Taxa analyzed**: *Ancistrus* cf. *dubius* Eigenmann & Eigenmann, 1889, and other not identified *Ancistrus* species – UFSCar / PPGGEv / CNPq / CAPES

II.2.5. MSc Dissertation by Ernani de Oliveira Mendes Neto (2008): Estudos citogenéticos em algumas espécies de Loricariidae (Teleostei, Siluriformes) da região de transposição do rio Piumhi para o rio São Francisco / Cytogenetic studies in Loricariidae species (Teleostei, Siluriformes) from the transposition region of the Piumhi River into the São Francisco River. **Taxa analyzed**: *Hypostomus regani, Hypostomus* sp.1*, Hypostomus* sp. 2, *Rineloricaria* cf. *latirostris* – UFSCar / PPGGEv / FAPESP

II.2.6. PhD Thesis by Marceleia Rubert (2011): Estudos citogenéticos em espécies das tribos Hipostomini e Ancistrini (Loricariidae, Hypostominae) / Cytogenetic studies in Hipostomini and Ancistrini species (Loricariidae, Hypostominae). **Taxa analyzed**: *Ancistrus brevipinnis* (Regan, 1904), *A. multispinis* (Regan, 1912), *Hemiancistrus punctulatus* Cardoso & Malabarba, 1999, *Hypostomus albopunctatus*, *H. cochiodon* Kner, 1854, *H. commersoni* Valenciennes, 1836, *H. heraldoi* Zawadzki, Weber & Pavanelli, 2008, *H. hermanni* (Ihering, 1905), *H. iheringii* (Regan, 1908), *H. mutucae* Knaack, 1999, *H. nigromaculatus* (Schubart, 1964), *H. paulinus* (Ihering, 1905), *H.* aff. *paulinus*, *H. regani*, *H. strigaticeps* (Regan, 1908) – UFSCar / PPGGEv / CAPES / CNPq

II.2.7. MSc Dissertation by Josiane Baccarin Traldi (2012): Citogenética comparativa em espécies de *Hypostomus* (Siluriformes, Loricariidae, Hypostominae). Contribuição da fração repetitiva do genoma para a diversidade cromossômica do grupo / Comparative cytogenetics in *Hypostomus* species (Siluriformes, Loricariidae, Hypostominae). Contribution of the repetitive genomic fraction to chromosomal diversity. **Taxa analyzed**: *H. ancistroides*, *H. iheringii*, *H. nigromaculatus*, *H. tapijara* Oyakawa, Akama & Zanata, 2005 – UFSCar / PPGGEv / FAPESP

II.2.8. PhD Thesis by Daniel Rodrigues Blanco (2012): Estudos citogenéticos clássicos e moleculares em espécies do gênero *Harttia* (Siluriformes, Loricariidae), com enfoque no papel dos DNAs repetitivos na evolução cariotípica do grupo / Conventional and molecular cytogenetic studies in *Harttia* species (Siluriformes, Loricariidae), focusing on the role of repetitive DNAs in the karyotypic evolution. **Taxa analyzed**: *H. loricariformes* Steindachner, 1877, *H. longipinna* Langeani, Oyakawa & Montoya-Burgos, 2001, *H. kronei* Miranda Ribeiro, 1908, *H. gracilis* Oyakawa, 1993, *H. punctata* Rapp Py-Daniel & Oliveira, 2001, *H. torrenticola* Oyakawa, 1993, *H. carvalhoi* Miranda Ribeiro, 1939 – UFSCar / PPGGEv / FAPESP

II.3. Family Auchenipteridae

II.3.1. MSc Dissertation by Roberto Laridondo Lui (2010): Análises comparativas citogenéticas e do DNA mitocondrial em *Parauchenipterus galeatus* Bleeker, 1862 (Siluriformes, Auchenipteridae) coletados no alto rio Paraná, no alto rio São Francisco e no rio Piumhi: um enfoque biogeográfico / Cytogenetic and mtDNA comparative analyses in *Parauchenipterus galeatus* Bleeker, 1862 (Siluriformes, Auchenipteridae) from the upper Paraná, Upper São Francisco and Piumhi Rivers: a biogeographical focus.**Taxon analyzed**: *Trachelyopterus galeatus* (Linnaeus, 1766) [cited as *P. galeatus* (Linnaeus, 1766)] – UFSCar / PPGGEv / FAPESP

II.3.2. PhD Thesis by Roberto Laridondo Lui (2012): Estudos evolutivos em Auchenipteridae (Siluriformes): citogenética, DNA mitocondrial e DNA satélite / Evolutionary studies in Auchenipteridae (Siluriformes): cytogenetics, mtDNA and satellite DNA.**Taxa analyzed**: *Ageneiosus inermis* (Linnaeus, 1766), *Glanidium ribeiroi* (Haseman, 1911), *Trachelyopterus galeatus* (cited as *Parauchenipterus galeatus*), *Trachelyopterus striatulus* (Steindachner, 1877) [cited as *Parauchenipterus striatulus* (Steindachner, 1877)], *Trachelyopterus* sp., *T. neivai* (Ihering, 1930), *Tatia jaracatia* Pavanelli & Bifi, 2009- UFSCar / PPGGEv / FAPESP

III. ORDER PERCIFORMES

III.1. Family Cichlidae

III.1.1. MSc Dissertation by Eliana Feldberg (1983): Estudos citogenéticos em 10 espécies da família Cichlidae (Pisces, Perciformes) / Cytogenetic studies in ten Cichlidae species (Pisces, Perciformes). **Taxa analyzed**: *Astronotus ocellatus* (Agassiz, 1831), *Cichlasoma facetum* (Jenyns, 1842), *Chaetobranchopsis australe* Eigenmann & Ward, 1907, *Crenicichla lacustris* (Castelnau, 1855), *C. lepidota* Keckel,1840, *C. vittata* Heckel, 1840, *C. semifasciata* (Heckel, 1840) (cited as *Batrachops semifasciatus* Heckel, 1840), *Geophagus brasiliensis* (Quoy & Gaimard, 1824), *G. surinamensis* (Block, 1791), *Gymnogeophagus balzanii* (Perugia, 1891) -UFSCar / PPGERN / CAPES

III.2. Family Serranidae

III.2.1. MSc Dissertation by Cecilia Texeira Aguilar (1993): Estudos citogenéticos em peixes da família Serranidae (Osteichthyes- Perciformes) ocorrentes na Baía de Guanabara – RJ / Cytogenetic studies in fishes of the family Serranidae (Osteichthyes-Perciformes) from the Guanabara Bay – RJ. **Taxa analyzed**: *Diplectrum radiale* (Quoy & Gaimard, 1824), *D. formosum* (Linnaeus, 1766), *Epinephelus marginatus* (Lowe, 1834) (cited as *Epinephelus guaza*; not of Linnaeus, 1758), *Mycteroperca rubra* (Bloch,1793), *Serranus flaviventris* (Cuvier, 1829) – UFRJ / PPGCB – Gene / CAPES

III.3. Family Pomacentridae

III.3.1. PhD Thesis by Wagner Franco Molina (2000): Análise da diversidade genética na famíla Pomacentridae (Pisces, Perciformes), utilizando métodos combinados de citogenética, marcadores moleculares e morfometria / Analysis of the genetic diversity in the family Pomacentridae (Pisces, Perciformes), employing cytogenetic, molecular and morphometric methods. **Taxa analyzed**: *Stegastes fuscus* (Cuvier, 1830), *S. variabilis* (Castelnau, 1855), *S. leucostictus* (Müller & Troschel, 1848), *S. pictus* (Castelnau, 1855), *S. rocasensis* (Emery, 1972), *S. sanctipauli* Lubbock & Edwards, 1981, *Abudefduf saxatilis* (Linnaeus, 1758), *Chromis multilineata* (Guichenot, 1853), *C. insolata* (Cuvier, 1830), *C. flavicauda* (Günther, 1880), *Microspathodon chrysurus* (Cuvier, 1830), *Amphiprion frenatus* Brevoort, 1856 – UFSCar / PPGGEv / CAPES

IV. ORDER OSTEOGLOSSIFORMES

IV.1. Family Arapaimidae

IV.1.1. PhD Thesis by Débora Karla Marques (2003): Caracterização genética do pirarucu, *Arapaima gigas* (Teleostei, Arapaimidae) / Genetic characterization of the pirarucu, *Arapaima gigas* (Teleostei, Arapaimidae). **Taxon analyzed**: *A. gigas* (Schinz, 1822) – UFSCar / PPGGEv

V. MISCELANEOUS GROUPS

V.1. MSc Dissertation by José das Neves Falcão (1983): Estudos citogenéticos em Acestrorhynchinae e Cynopotaminae (Pisces, Characidae) / Cytogenetic studies in Acestrorhynchinae and Cynopotaminae (Pisces, Characidae). **Taxa analyzed**: Order Characiformes – Family Acestrorhynchidae: *Acestrorhynchus altus* Menezes, 1969, *A. lacustris* (Lütken, 1875), Family Characidae: *Galeocharax knerii* (Steindacher, 1879), *Oligosarcus hepsetus* (Cuvier, 1829), *O. jenynsii* (Günther, 1864), *Oligosarcus* sp., *O. pintoi* Amaral Campos, 1945 (cited as *Paroligosarcus pintoi*) – USP / PPGCB – Gene / CAPES

V.2. PhD Thesis by Mario Jorge Ignacio Brum (1994): A evolução cariotípica dos teleósteos marinhos e suas correlações com a filogenia deste grupo / Karyotype evolution of marine teleosts and its correlation with the phylogeny of the group. **Taxa analyzed** – Order Clupeiformes – Family Clupeidae: *Brevoortia aurea* (Spix & Agassiz, 1829); Order Perciformes–Family Haemulidae: *Orthopristis ruber* (Cuvier, 1830); Family Blenniidae: *Scartella cristata* (Linnaeus, 1758) – Order Tetraodontiformes – Family Tetraodontidae: *Sphaeroides greeleyi* Gilbert, 1900 – UFSCar / PPGGEv

V.3. MSc Dissertation by Lilian Cristina Jorge (1995): Estudos citogenéticos comparativos de algumas espécies de peixes da região de Corrientes (Argentina) com as do Alto Paraná / Comparative cytogenetic studies of some fish species from the

Corrientes region (Argentina) with those of the upper Paraná River basin.**Taxa analyzed** – Order Characiformes – Family Characidae: *Astyanax bimaculatus*; Family Parodontidae: *Apareiodon affinis*; Family Anostomidae: *Leporinus obtusidens*; Family Erythrinidae: *Hoplias malabaricus*, *Hoplerythrinus unitaeniatus* – UFSCar / PPGGEv / FAPESP.

V.4. MSc Dissertation by Margareth Maria de Oliveira Correa (1995): Contribuição à citotaxonomia dos Scorpaeniformes (Osteichthyes-Teleostei). Estudos citogenéticos em espécies do litoral do Rio de Janeiro, Brasil / Contribution to the cytotaxonomy of Scorpaeniformes (Osteichthyes-Teloestei). Cytogenetic studies in coastal species from Rio de Janeiro, Brazil. **Taxa analyzed –** Order Scorpaeniformes–Family Dactylopteridae: *Dactylopterus volitans* (Linnaeus, 1758); Family Scorpaenidae: *Scorpaena brasiliensis* Cuvier, 1829; *S. isthmensis* Meek & Hildebrand, 1928; Family Triglidae: *Prionotus punctatus* (Block, 1793) – UFRJ / PPGCB-Ecol / CNPq

V.5. PhD Thesis by Carlos Suetoshi Miyazawa (1997): Citogenética de caracídeos do rio Paraguai. Análises citotaxonômica-evolutivas e considerações biogeográficas / Cytogenetics of characids from the Paraguay River. Cytotaxonomic and evolutionary analyses and biogeographical considerations. **Taxa analyzed**: Order Characiformes – Family Acestrorhynchidae: *Acestrochynchus pantaneiro* Menezes, 1992; Family Serrasalmidae: *Metynnis maculatus* (Kner, 1858), *Myleus levis* (Eigenmann & McAtee, 1907) (cited as *Myloplus levis*); Family Characidae: *Poptella paraguayensis* (Eigenmann,1907), *Tetragonopterus argenteus* (Cuvier, 1816), *Roeboides* sp.; *Astyanax* cf. *abramis* (Jenyns, 1842), *Markiana nigripinnis* (Perugia, 1891), *Gymnocorymbus ternetzi* (Boulenger, 1895), *Moenkhausia dichroura* (Kner, 1858); Family Triportheidae: *Triportheus* sp., Family Gasteropelecidae*: Thoracocharax stellatus* (Kner, 1858) – UFSCar / PPGGEv / CAPES

V.6. PhD Thesis by Paulo Cesar Venere (1998): Diversificação cariotípica em peixes do médio rio Araguaia, com ênfase em Characiformes e Siluriformes (Teleostei, Ostariophysi) / Karyotype diversification in fishes from the middle Araguaia River, with emphasis on Characiformes and Siluriformes (Teleostei, Ostariophysi). **Taxa analyzed**: Order Characiformes – Family Anostomidae: *Leporinus friderici*, *L. trifasciatus* Steindachner, 1876, *Leporinus* sp.*, Leporinus* aff. *brunneus* Myers, 1950, *Laemolyta petiti* Géry, 1964; Family Prochilodontide: *Prochilodus nigricans*; Family Chilodontidae: *Caenotropus labyrinthicus* (Kner, 1858); Family Curimatidae: *Steindachnerina amazônica* (Steindachner, 1911), *S. gracilis* Vari & Williams Vari, 1989, *Curimata inornata* Vari, 1989, *Psectrogaster amazônica* Eigenmann & Eigenmann, 1889; Family Hemiodontidae: *Hemiodus* aff. *ternetzi* Myers, 1927, *H. unimaculatus* (Bloch, 1794), *Bivibranchi avelox* (Eigenmann & Myers, 1927); Family Characidae: *Roeboides* sp., *Galeocharax gulo* (Cope, 1870), *Exodon paradoxus* Müller & Troschel, 1844; Order Siluriformes – Family Doradidae: *Hassar wilderi* Kindle, 1895, *Leptodoras acipenserinus* (Günther, 1868), *Opsodoras* sp., *Rinodoras* sp.; Family Auchenipteridae: *Trachelyopterus* aff. *galeatus* (cited as *Parauchenipterus* aff. *galeatus*); Family Callichthyidae: *Megalechis thoracata* (Valenciennes, 1840),

cited as *Megalechis personata* (Ranzani, 1841); Family Gymnotidae: *Gymnotus* aff. *carapo* Linnaeus, 1758 – UFSCar / PPGGEv / CAPES

V.7. MSc Dissertation by Paulo Roberto Antunes de Mello Affonso (2000): Caracterização citogenética de peixes de recifes de corais das famílias Pomacanthidae e Chaetodontidae (Perciformes) / Cytogenetic characterization of coral reef fishes of the Pomacanthidae and Chaetodontidae families (Perciformes). **Taxa analyzed**: Order Perciformes – Family Pomacanthidae: *Centropigea urantonotus* Burgess, 1974, *Holocanthus ciliaris* (Linnaeus, 1758), *H. tricolor* (Block, 1795), *Pomacanthus arcuatus* (Linnaeus, 1758), *P. paru* (Block, 1787); Family Chaetodontidae: *Chaetodon striatus* Linnaeus, 1758 – UFSCar / PPGGEv / CNPq

V.8. MSc Dissertation by Marilza Barbosa de Almeida Marques (2002): Estudos citogenéticos em *Conorhynchus conirostris* e *Lophiosilurus alexandri* (Siluriformes), espécies endêmicas do rio São Francisco / Cytogenetic studies in *Conorhynchus conirostris* and *Lophiosilurus alexandri* (Siluriformes), endemic species from the São Francisco River.**Taxa analyzed**: Order Siluriformes – Family Pimelodidae: *C. conirostris* (Valenciennes, 1840); Family Pseudopimelodidae: *L. alexandri* Steindachner, 1876 – UFSCar / PPGGEv

V.9. MSc Dissertation by Karine Frehner Kavalco (2003): Contribuição citogenética à análise da biodiversidade da ictiofauna das nascentes do rio Paraitinga. / Cytogenetic contribution to the biodiversity analysis of the fish fauna from the headwaters of the Paraitinga River. **Taxa analyzed**: Order Siluriformes – Family Loricariidae: *Harttia loricariformes* Steindachner, 1877, *Neoplecostomus microps* (Steindachner, 1877), *Hypostomus affinis* (Steindachner, 1877), *Upsilodus* sp.; Order Characiformes – Family Characidae: *Astyanax scabripinnis*, *A. parahybae* Eigenmann, 1908, *A. intermedius* Eigenmann, 1908, *A. giton* Eigenmann, 1908, *Oligosarcus hepsetus* – UFSCar / PPGGEv / FAPESP

V.10. PhD Thesis by Liano Centofante (2003): Citogenética comparativa entre ictiofaunas isoladas por um divisor de águas em regiões limítrofes de duas bacias hidrográficas na Serra da Mantiqueira / Comparative cytogenetics of fish fauna from neighboring regions of two hydrographic basins isolated by a watershed in the Serra da Mantiqueira. **Taxa analyzed**: Order Siluriformes – Family Loricariidae: *Harttia carvalhoi* Miranda Ribeiro, 1939; Family Heptapteridae: *Rhamdia* sp.; Order Characiformes – Family Characidae – *Astyanax parahybae*, *A. fasciatus*, *Hyphessobrycon anisitsi* (Eigenmann, 1907); Family Parodontidae: *Parodon nasus* (cited as *P. tortuosus*), *P. moreirai* Ingenito & Buckup, 2005 (cited as *Parodon* sp); Family Crenuchidae: *Characidium gomesi* Travassos, 1956, *C.* cf. *zebra* Eigenmann, 1909, *C. lauroi* Travassos, 1949, *C.* cf. *alipioi* Travassos, 1955 – UFSCar / PPGGEv / CNPq / CAPES

V.11. MSc Dissertation by Caroline Garcia (2005): Contribuições aos estudos citogenéticos em algumas espécies de cinco famílias de Siluriformes do rio São Francisco / Contributions to cytogenetics of some species of three Siluriformes families from the São Francisco River. **Taxa analyzed**: Order Siluriformes – Family Auchenipteridae: *Trachelyopterus galeatus* (cited as *Pauracheniptеrus galeatus*), *T. leo-*

pardinus (Borodin, 1927) cited as *Pauracheniptérus leopardinus* (Borodin, 1927); Family Doradidae: *Fransciscodoras marmoratus* (Lütken, 1874); Family Heptapteridae: *Rhamdia quelen*; Family Pimelodidae: *Pimelodus fur* (Lütken, 1874), *P. maculatus* Lacepède, 1803, *Pimelodus* sp., *Zungaru zungaru* (Humboldt, 1821) cited as *Pseudopimelodus zungaru* (Humboldt, 1821) – UFSCar / PPGGEv / FAPESP

V.12. PhD Thesis by Marcelo Ricardo Vicari (2006): Diversidade de peixes residentes em cabeceiras de rios. Uma abordagem cromossômica em três diferentes biomas aquáticos da região Sul do Brasil / Fish diversity from river headwaters. A chromosomal approach in three biomes from South Brazil. **Taxa analyzed**: Order Siluriformes – Family Callichthyidae: *Corydoras paleatus* (Jenyns, 1842), *C. ehrhardti* Steindachner, 1910; Order Characiformes – Family Parodontidae: *Apareiodon* sp.; Family Characidae: *Astyanax scabripinnis*, *A. janeiroensis* Eigenmann, 1908; Family Crenuchidae: *Characidium* cf. *gomesi*; Order Perciformes – Family Cichlidae: *Geophagus brasiliensis*, *Australoheros facetus* (Jenyns, 1842) cited as *Cichlasoma facetum* (Jenyns, 1842) – UFSCar / PPGGEv / FAPESP

V.13. MSc Dissertation by Maressa Ferreira Neto (2008): Análise citogenética em algumas espécies de peixes de uma região divisora de águas entre riachos de bacias hidrográficas distintas **/** Cytogenetic analysis in fish species from a dividing water region of streams belonging to different river basins. **Taxa analyzed**: Order Characiformes – Family Characidae: *Astyanax altiparanae, A. fasciatus***,** *Moenkausia sancta filomenae*; Family Curimatidae: *Cyphocarax modestus*; Family Prochilodontidae: *Prochilodus lineatus*; Order Gymnotiformes – Family Gymnotidae: *Gymnotus carapo* Linnaeus, 1758; Family Sternopygidae: *Eigenmannia* sp.; Order Perciformes – Family Cichlidae: *Geophagus brasiliensis* – UFSCar / PPGGEv / CAPES / CNPq

V.14- PhD Thesis by Elisangela Bellafronte da Silva (2009): Citogenética clássica e molecular em peixes Neotropicais. Estudos comparativos entre bacias hidrográficas com ênfase em região de transposição de rio / Conventional and molecular cytogenetics in Neotropical fishes. Comparative studies among river basins with emphasis on a river transposition region. **Taxa analyzed**: Order Gymnotiformes – Family Gymnotidae: *Gymnotus carapo*, *G. silvius* Albert & Fernandes-Matioli, 1999; Family Sternopygidae: *Eigenmannia virescens* (Valenciennes, 1836), *Eigenmania* sp. – UFSCar / PPGGEv / CNPq / CAPES

V.15. PhD Thesis by Daniel Luis Zanella Kantek (2010): Citogenética de espécies de Siluriformes da região de transposição do rio Piumhi (MG) / Cytogenetics of Siluriformes species from the transposition region of the Piumhi River (MG). **Taxa analyzed**: Order Siluriformes – Family Auchenipteridae: *Trachelyopterus galeatus* (cited as *Parauchenipterus galeatus*); Family Pimelodidae: *Pimelodus pohli* Ribeiro & Lucena, 2006; Family Heptapteridae: *Imparfinis schubarti* (Gomes, 1956); *Cetopsorhamdia iheringi* Schubart & Gomes, 1959, *Pimelodella vittata* (Lütken, 1874), *Rhamdia* sp. A, *Rhamdia* sp. B, *Rhamdiopsis* cf. *microcephala* (Lütken, 1874), Family Trichomycteridae: *Trichomycterus brasiliensis* Lütken, 1874 – UFSCar / PPGGEv / CNPq / CAPES

Final remarks

Two general trends were found among the Neotropical fishes regarding the karyotype evolution. In fact, a significant number of families were characterized by conservative karyotypes, in contrast to others with highly divergent ones. Parodontidae, Anostomidae and Prochilodontidae species, for example, exhibit relatively homogeneous karyotypes at the macrostructural level, contrasting with the high chromosomal diversity found among Erythrinidae and Characidae species (Bertollo et al. 1986). It is noteworthy that karyotype features appear to be correlated with their lifestyle and ecological habits, since more dispersive and migratory species usually disclose more stable karyotypes when compared to those with low vagility and organized in small local populations (Bertollo et al. 1986; Blanco et al.2011; Oliveira et al. 2015). Indeed, many local populations were evidenced as having particular karyotypes, pointing to a large number of species complexes and the cryptic biodiversity present in the Neotropical fish fauna, as especially highlighted in the Characidae and Erythrinidae families (Moreira-Filho and Bertollo1991; Bertollo 2007; Cioffi et al. 2012a). In fact, many sympatric, or even syntopic, karyomorphs do not indicate hybridization at the chromosomal level, indicating the absence of gene flow among them and, consequently, corroborating the status of species complexes for some current nominal species (Bertollo et al. 2000).

Reports on chromosomal polymorphisms (Giuliano-Caetano and Bertollo 1988; Vicari et al. 2003; Pazza et al. 2006, 2008; Mariotto et al. 2009), natural triploidy (Morelli et al. 1983; Venere and Galetti Jr. 1985; Giuliano-Caetano and Bertollo 1990; Centofante et al. 2001; Garcia et al. 2003) and broad karyotype evolution by centric fissions (Feldberg et al. 1993), were also emphasized for distinct fish groups. Noteworthy is also the cytogenetic contribution for biogeographical analyzes, clarifying the current fish fauna distribution in some important Brazilian river basins. In this sense, native species, as well as invasive ones due to dispersal events or breakdown of geographic isolation, were clearly identified by chromosomal investigations (Peres et al. 2009; Blanco et al. 2010; Silva et al. 2010; Perez et al. 2012). As a significant example, *Astyanax bimaculatus* from two important Brazilian watersheds, namely the São Francisco and Grande rivers share similar morphological characteristics. However, specimens from each one of such rivers were well characterized by their particular chromosomal features. In the early 1960s, a tributary of the Grande River was artificially transposed into the São Francisco river basin, with the consequent breakdown of the geographic isolation of their respective fish fauna. As a consequence, cytogenetic investigation was able to identify representatives of *A. bimaculatus* from both basins living in sympatry in the transposition region, as well as individuals with intermediate karyotypes in view of the resulting secondary hybrid zone in such region (Peres et al. 2012).

Over the years, a particular emphasis has been directed on the characterization and the evolutionary process of sex chromosomes. A larger number of Neotropical fish species with well differentiated sex chromosomes occur in comparison to other world regions (Moreira-Filho et al. 1993), carrying simple (ZZ/ZW, XX/XY) and multiple

($X_1X_1X_2X_2/X_1X_2Y$, XX/XY_1Y_2, ZZ/ZW_1W_2) sex chromosome systems (Centofante et al. 2002; Cioffi et al. 2012b), in addition to some others disclosing a nascent or early stage of differentiation (Cioffi and Bertollo 2010; Freitas et al. in press). Usually, sex chromosomes occur as a particular feature for some species within a specific fish group, as exemplified in the Erythrinidae, Parodontidae, Anostomidae and Crenuchidae families (Galetti Jr. et al. 1981, 1995; Moreira-Filho et al. 1985, 1993; Molina et al. 1998; Centofante et al. 2001, 2003; Bertollo et al. 2000; Bertollo 2007; Vicari et al. 2008; Cioffi et al. 2013). As a singular exception, all species of the *Triportheus* genus (Triportheidae) share a same ZZ/ZW sex chromosome system (Artoni et al. 2001, 2002; Diniz et al. 2008), constituting a special model to investigate the evolution of the sex chromosomes among lower vertebrates. The modern molecular cytogenetics was a key step for understanding the evolutionary process of the sex chromosomes among fishes. This way, the significative role of several classes of repetitive DNAs in the differentiation path of the sex pair, both at its inicial stage (Cioffi and Bertollo 2010; Freitas et al. 2017) or more advanced ones (Cioffi et al. 2010, 2011a, b, 2012b; Yano et al. 2014a, b), was clearly highlighted. Notably, whole chromosome painting (WCP) and comparative genomic hybridization (CGH) were able to demonstrate that fish sex chromosomes can have an independent origin even among closely related species (Cioffi et al. 2011c, d; 2013) or, alternatively, a common origin within particular monophyletic groups (Yano et al. 2016).

Besides sex chromosomes, supernumerary or B chromosomes comprise another special feature that stands out in the Neotropical fishes. Such additional elements can be i) as large as the biggest chromosome pair of the karyotype, ii) medium-sized, iii) very small iv) or even characterized as microchromosomes. Two particular models, represented by *Astyanax scabripinnis* and *Prochilodus lineatus*, have been subjected to continuous analyses over years. *A. scabripinnis* has some morphologically differentiated B chromosomes, although a large and similar in size to the first chromosome pair of the karyotype is the most frequent one (Moreira-Filho et al. 2004). Its origin as an isochromosome was demonstrated by both standard and molecular cytogenetic, including meiotic data (Vicente et al. 1996; Mestriner et al. 2000). A continuous population analysis showed that Bs display a particular dynamism related to environmental and sex conditions in *A. scabripinnis*. Indeed, it is noteworthy their gradual decrease in frequency from higher to lower altitudes, until the complete absence in the latter ones (Néo et al. 2000). In addition, an evident sex ratio distortion is associated with these chromosomes. In fact, the mean number of Bs in males is only about 27% of the female one, which matches the male population frequency (Vicente et al. 1996), suggesting that B chromosomes may play a role on sex determination in this species.

Constrasting with *A. scabripinnis*, *P. lineatus* bears a number of very small B chromosomes (Pauls and Bertollo 1983), which also have an intraspecific origin as indicated by molecular cytogenetic and chromosomal banding (Jesus et al. 2003; Artoni et al. 2006). Remarkably, the frequency of these chromosomes was changed over years in close association with their transmission dynamics. In this sense, the average number of Bs increased twice along a time period indicating an accumulation mechanism, but

without evidences of additional changes after that. Significantly, the mitotic instability of Bs declined almost 400 times during this same period, reaching a stable transmission. This way, it is likely that the mitotic stabilization was a key process for neutralizing the accumulation process (Cavallaro et al. 2000).

Nowadays, many of such issues so far investigated, in addition to additional approaches on fish biology, are going in advancing in the light of chromosomal, cytogenomic and molecular methodologies currently available. It is hoped that these procedures can provide additional and important advances for the Neotropical fish fauna evolutionary history.

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RESEARCH ARTICLE

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Meiotic behaviour and its implication on species inter-relationship in the genus *Curcuma* (Linnaeus, 1753) (Zingiberaceae)

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Abstract

In this paper, detailed meiotic analysis was investigated in seven species of *Curcuma* (Linnaeus, 1753) which can contribute significantly to our understanding about species inter-relationship, speciation and evolution. The species were divided into two groups viz., Group I having 2n = 42 (*C. comosa* Roxburgh, 1810, *C. haritha* Mangaly & M.Sabu, 1993, *C. mangga* Valeton & Zijp, 1917, and *C. motana* Roxburgh, 1800) and Group II with 2n = 63 (*C. caesia* Roxburgh, 1810, *C. longa* Linnaeus, 1753 and *C. sylvatica* Valeton, 1918). Both groups display varying degree of chromosome associations. Group I species showed the prevalence of bivalents, however occasional quadrivalents besides univalents were also encountered. About 48% of the PMCs analyzed in *C. mangga* showed 21 bivalents (II) meiotic configurations, 32% in *C. comosa* and 16% in *C. haritha.* Group II species as expected showed the presence of trivalents besides bivalents, univalents and quadrivalents. About 32% of the PMCs analyzed at MI in *C. sylvatica* showed 21 trivalents (III) meiotic configurations, 24% in *C. longa* and 8% in *C. caesia.* Overall, low frequency of multivalent associations as compared to bivalents indicates that *Curcuma* is an allopolyploid complex. Moreover, x = 21 is too high a basic number, therefore, we suggest that the genus *Curcuma* has evolved by hybridization of species with different chromosome numbers of 2n = 24 and 18, resulting in a dibasic amphidiploid species.

Keywords

Polyploidy, amphidiploid, inter-specific crosses, diversification

Introduction

The genus *Curcuma* Linn. belonging to the tribe Zingibereae of the family Zingiberaceae consists of about 120 species and is pan-tropical in distribution (Kress et al. 2002, Škorničková et al. 2007, Záveská et al. 2012). It contains many taxa with multifaceted uses and quite a few species of *Curcuma* (e.g. *C. amada*, *C. caesia*, *C. longa,* etc.) are used as spice, dye, medicine, cosmetics, ornamental and as a source for starch (Sasikumar 2005,Velayudhan, 2012).

Curcuma, a rhizomatous, perennial and herbaceous group of plant displays a great degree of diversity in ploidy levels which is evident from earlier cytogenetical studies wherein various chromosome numbers of 2n = 22, 42, 63, 77, 105, etc., have been reported. Moreover, continuous dispute concerning the basic chromosome number in *Curcuma* (x = 7, 8, 16 and 21) has been highlighted in early cytological studies of Raghavan and Venkatasubban (1943), Sharma and Bhattacharya (1959), Ramachandran (1961), Islam (2004), Škorničková et al. (2007). Whilst a lot of information on the somatic chromosome number is available for the genus *Curcuma*, essential information about the homology among the chromosome complements and level of polyploidy has yet to be investigated.

Meiosis, a highly conserved and specialized process in eukaryotes, not only generates genetic variability but also ensures gamete viability and constancy of ploidy levels (Pagliarini 2000, Hamant et al. 2006, Kumar and Singhal 2011, Brownfield and Köhler 2011). However, disruption of meiosis as well as pre- and post- meiotic events can have a severe effect on the genetic stability and viability of the gametes (Brownfield and Köhler 2011). Moreover, the degree of association and behaviour of chromosome pairing, chiasma distribution and its frequencies, disjunction of chromosomes in anaphase I/II can also provide significant insight on speciation and structural details of genomic organization and species interrelationships (Sharma et al. 2011). Chromosome pairing, an important feature of meiosis, has often been used to infer genome relationship in hybrids and polyploid species (Grandont et al. 2013). Such studies might also contribute to the better understanding of cytological evolution of species which can be utilized for future genetic improvement and conservation of the genetic resources (Kumar and Singhal 2011). However, detailed studies on male meiosis are very much limited in the genus *Curcuma* except for a few reports of Ramachandran (1961), Nambiar (1979) and Puangpairote et al. (2016). The possible reason may be due to rare flowering of the plants under non-optimal environment and factors like inherent difficulty in obtaining good analyzable cytological preparations, small chromosome size and stainability (Puangpairote et al. 2016).

In this context, seven species of *Curcuma*: *C. comosa* Roxburgh, 1810, *C. haritha* Mangaly & M.Sabu, 1993, *C. mangga* Valeton & Zijp, 1917, *C. montana* Roxburgh, 1800, *C. caesia* Roxburgh, 1810, *C. longa* Linnaeus, 1753 and *C. sylvatica* Valeton, 1918, were taken up for the present investigation for analysis of meiotic pairing behaviour

in order to find evidence on species inter-relationship, speciation and evolution. From our previous investigations on chromosome count, the somatic chromosome number in *C. comosa, C. haritha, C. mangga* and *C. montana* was observed to be 2n = 42 while 2n = 63 was recorded in *C. caesia, C. longa* and *C. sylvatica* (Lamo and Rao 2014, 2017).

Material and methods

For the present investigation, *Curcuma* germplasm along with their specimen voucher numbers were obtained from Indian Institute of Spices Research, Kozhikode. Flower buds were obtained from the plants growing in polyhouse conditions at the Department of Biotechnology and Bioinformatics, North-Eastern Hill University, Shillong.

Flower buds of appropriate size were fixed in freshly prepared Carnoy's solution (1:3 glacial acetic acid: 95% ethanol) for 4 days at room temperature and stored in 70% ethanol at 4°C. Anthers were squashed in 2% aceto-carmine solution and in some cases ferric chloride solution was used as mordant. The slides were examined and photographed using Leica DM 4000 B microscope attached to Leica CCD camera at ×1000 magnification. For meiotic analysis each preparation was determined by microscopy as well as photomicrographs. On an average 25 PMCs/species were used for detailed analysis at diplotene, diakinesis and/or metaphase I.

The terminalization coefficient was calculated using the following formula:

$$\textbf{Terminalization coefficient} = \frac{\textbf{Total number of terminalized chiasma}}{\textbf{Total number of chiasma observed}}$$

Results

Group I (2n = 42)

C. comosa

Chromosome associations at diplotene, diakinesis and metaphase I (MI) were characterised by both bivalents and univalents besides quadrivalents (Fig. 1a–d). About 32% of the PMCs were characterised by 21 bivalents (21II). Bivalents ranged from 13–21 with a mean value of 18.24. The bivalents showed both ring and rod association which ranges from 2–13 and 7–15 with a mean value of 7.44 and 10.80 respectively (Table 1). Quadrivalents ranged from 0 to 2 with a mean value of 0.68, whereas univalents ranged from 0–8 with a mean value of 2.80. No trivalent associations were encountered in any of the PMCs analysed. The total number of chiasmata observed was 796 out of which 619 were terminalized and 177 were unterminalized resulting in a terminalization coefficient of 0.78.

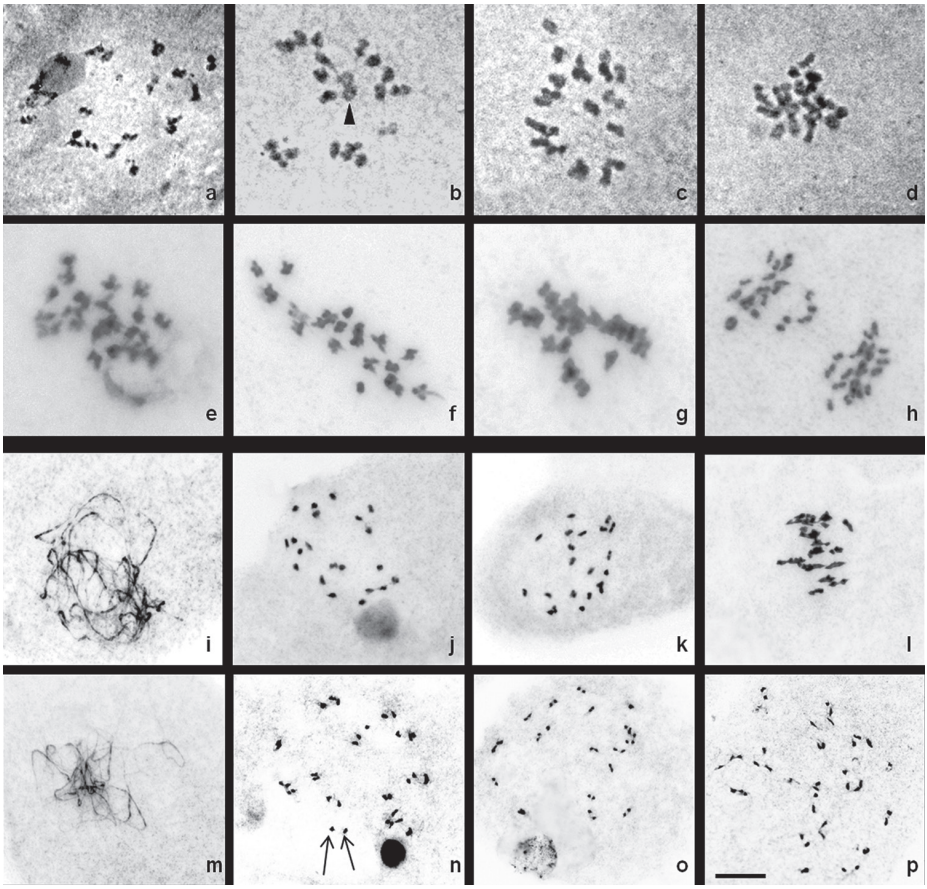


Figure 1. Male meiosis in group I. **a–d** *C. comosa*: **a** diplotene **b** diakinesis **c–d** metaphase I **e–h** *C. haritha*: **e** diakinesis **f** metaphase I **g–h** anaphase I **i–l** *C.mangga*: **i** pachytene **j** diplotene **k** diakinesis **l** metaphase I **m–p** *C. montana*: **m** pachytene **n** diplotene, **o–p** diakinesis; arrowhead showing multivalent and arrows showing univalents.Bar = 10 µm.

C. haritha

About 16% of the PMCs were characterised by the formation of 21II, while the remaining PMCs were characterised by both bivalent and multivalent associations besides univalents (Fig. 1e–h). The number of bivalents ranged from 13–21 with a mean value of 15.80 (Table 1). The ring bivalent ranges from 0–6 with a mean value of 3.20 and rod bivalents ranged from 11–12 with a mean value of 12.64. Quadrivalent associations ranged from 0 to 2 with a mean value of 0.52 and the total number of univalents was 208 with a mean value of 8.32. No trivalent associations were encountered in any of the PMCs analysed. The total number of chiasmata observed was 557 with a mean value of 22.28 (Table 2). The total number of terminalized chiasmata was 472 and unterminalized chiasmata were 85 yielding a terminalization coefficient of 0.85. About 72.73% and 27.27% of the PMCs analyzed showed 21:21 and 24:18 chromosome distributions at AI respectively.

Table 1. Mean number and range of associations at diplotene/diakinesis/metaphase-I in Curcuma species.

Species	IISR Voucher No.	Chromosome associations																	
		Bivalent									Univalent			Trivalent			Quadrivalent		
					Ring			Rod											
		No.	Mean ±SD	Range	No.	Mean ±SD	Range	No.	Mean ±SD	Range	No.	Mean ± SD	Range	No.	Mean ± SD	Range	No.	Mean ± SD	Range
C. comosa	644	456	18.24 ±2.31	13–21	186	7.44 ±3.44	2–13	270	10.80 ±2.25	7–15	70	2.80 ±2.58	0–8	–	–	–	17	0.68 ±0.63	0–2
C. haritha	1136	395	15.80 ±2.27	13–21	80	3.20 ±1.50	0–6	315	12.64 ±3.1	11–12	208	8.32 ±3.90	0–16	–	–	–	13	0.52 ±0.59	0–2
C. mangga	1049	487	19.48 ±1.56	17–21	171	6.84 ±2.27	2–11	316	12.64 ±2.10	9–15	16	0.64 ±0.95	0–2	–	–	–	15	0.64 ±0.95	0–2
C. montana	649	421	16.84 ±1.91	12–20	174	6.96 ±1.97	4–12	247	9.86 ±2.60	6–15	96	3.84 ±2.60	0–12	–	–	–	28	1.12 ±0.93	0–3
C. caesia	751	361	14.44 ±7.80	0–24	102	4.08 ±2.38	0–8	259	10.36 ±6.16	4–20	70	2.90 ±2.45	0–8	213	8.52 ±6.73	0–21	36	1.44 ±1.22	0–3
C. longa	Pratibha	304	12.16 ±8.84	0–24	178	7.12 ±5.43	0–17	126	5.04 ±3.89	0–13	105	4.20 ±3.77	0–14	246	9.84 ±7.44	1–21	31	1.24 ±0.88	0–2
C. sylvatica	526	424	16.96 ±12.25	0–29	119	4.76 ±4.01	0–11	305	12.20 ±9.21	0–24	33	1.32 ±1.81	0–8	213	8.52 ±8.85	0–21	14	0.56 ±0.87	0–3

Table 2. Mean number and range of chiasma, terminalization coefficient and percentage of pollen stainability in *Curcuma* species.

Species	No of cells analysed	Chiasma					Terminalization coefficient
		Total	Mean ± SD	Range	Terminalized ± SD	Unterminalized ± SD	
C. comosa	25	796	10.80±2.5	15–32	24.76±5.79	7.08±1.91	0.78
C. haritha	25	557	22.28±3.2.7	17–30	18.88±2.15	3.40±2.24	0.85
C. mangga	25	726	29.04±4.22	25–37	22.60±4.41	6.44±1.64	0.78
C. montana	25	718	28.72 ±3.61	28–40	23.72±5.56	5.92±2.38	0.82
C. caesia	20	1023	51.15±6.22	45–69	39.05±6.91	12.10±4.67	0.76
C. longa	25	676	27.04±19.62	0–49	19.76±14.68	7.28±5.34	0.73
C. sylvatica	28	1365	48.75 ±9.89	36–61	35.82±7.49	12.93±6.89	0.74

C. mangga

About 48% of the PMCs analysed showed 21II, while the rest showed a mix of both bivalent and multivalent associations besides univalents (Fig. 1i–l). The number of bivalents ranged from 17–21 with a mean value of 19.48 (Table 1). The ring bivalent ranged from 2–11 with a mean value of 6.84 and rod bivalents ranged from 9–15 with a mean value of 12.64. Quadrivalent associations observed ranged from 0–2 with a mean value of 0.64. Total number of univalents recorded was 16 with a mean value of 0.64. No trivalent associations were encountered in any of the cells analysed. The total number of chiasmata observed was 726 with a mean value of 29.04 ranging from 25–37 (Table 2). About 565 chiasmata were terminalized and 161 were unterminalized yielding a terminalization coefficient of 0.78.

C. montana

Detailed analysis at diplotene, diakinesis and metaphase showed that bivalents ranged from 12–20 with a mean value of 16.84 (Table 1; Fig. 1m–p). The number of ring bivalents ranged from 4–12 with a mean value of 6.96 and rod bivalents ranged from 6–15 with a mean value of 9.86. Quadrivalents ranged from 0 to 3 with a mean value of 1.12. Univalent lie in close proximity to each other and the total number of univalent recorded was 96 with a mean value of 3.84. No trivalent associations were encountered in any of the cells analysed. The total number of chiasmata observed was 718 with a mean value of 28.72 (Table 2). The total number of terminalized and unterminalized chiasmata was 593 and 148 respectively. Terminalization coefficient of 0.82 was being recorded.

Group II (2n = 63)

C. caesia

About 8% of the PMCs analysed showed trivalent associations (21III) while the rest showed both bivalent and multivalent associations along with univalents (Fig. 2a–d).

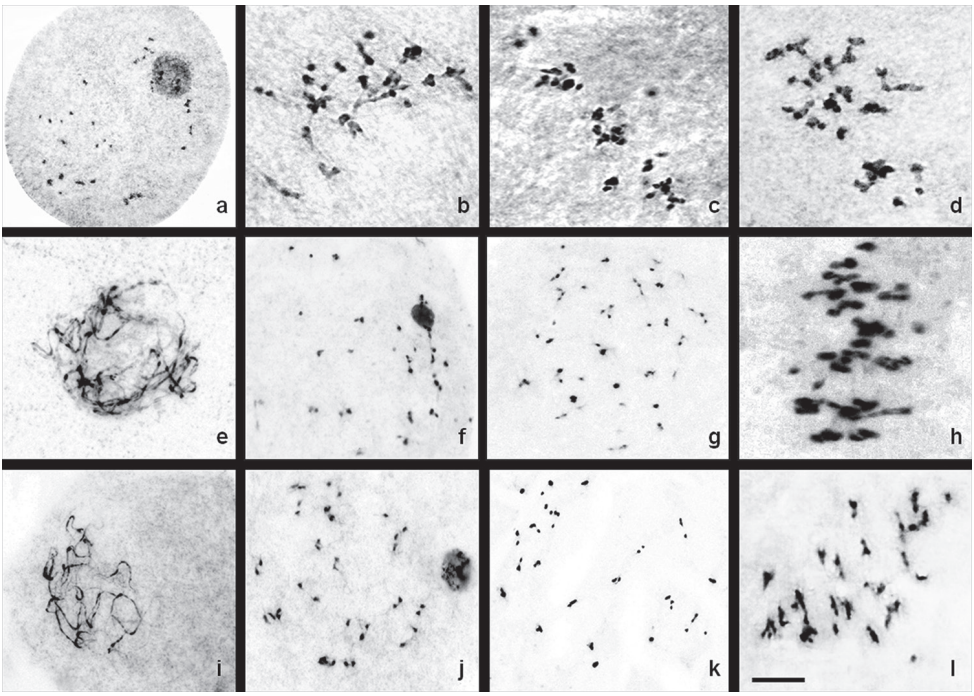


Figure 2. Male meiosis in Group II. **a–d** *C. caesia*: **a** diplotene **b** diakinesis **c–d** metaphase I **e–h** *C. longa*: **e** pachytene **f** diplotene **g** diakinesis **h** metaphase I **i–l** *C. sylvatica*: **i** pachytene **j** diplotene **k** diakinesis **l** metaphase I. Bar = 10 μm.

The number of bivalents ranged from 0–24 with a mean value of 14.44 (Table 1). The ring bivalent ranges from 0–8 with a mean value of 4.08 and rod bivalents ranged from 4–20 with a mean value of 10.36. Trivalents ranges from 0–21 with a mean value of 8.52 while quadrivalents ranged from 0 to 3 with a mean value of 1.44. The total number of univalent recorded was 70. The total number of chiasmata observed was 1023 with a mean value of 51.15 (Table 2). Out of the 1023 chiasmata observed, 781 were terminalized and 242 were unterminalized yielding a terminalization coefficient of 0.76.

C. longa

About 24% of the PMCs analysed showed trivalent associations (21III) and the rest showed the occurrence of both bivalents and multivalents (trivalent and quadrivalent) associations along with univalents (Fig. 2e–h). The number of bivalents ranged from 0–24 with a mean value of 12.16 (Table 1). The ring bivalent ranges from 0–17 with a mean value of 7.12 and rod bivalents ranged from 0–13 with a mean value of 5.04. Trivalents ranged from 1–21 with a mean value of 9.84. Quadrivalent associations ranged from 0 to 2 with a mean value of 1.24. The total number of univalents was 105.

Table 3. Percentage of chromosome associations during male meiosis in *Curcuma* species.

	C. comosa	*C. haritha*	*C. mangga*	*C. montana*	*C. caesia*	*C. longa*	*C. sylvatica*
Quadrivalents	3.13	2.11	2.89	5.14	5.30	4.52	2.05
Trivalents	–	–	–	–	31.32	35.86	31.14
Bivalents	83.98	64.12	94.02	77.23	53.09	44.31	61.99
Univalents	12.89	33.77	3.89	17.63	10.29	15.31	4.82

The total number of chiasmata recorded was 676 with a mean value of 27.04 ranging from 0–49 (Table 2). Out of 676 chiasmata 494 were terminalized and 182 were unterminalized yielding a terminalization coefficient of 0.73.

C. sylvatica

PMCs analysed showed 32% trivalent associations and the rest showed both bivalent and multivalent associations along with univalents (Fig. 2i–l). The number of bivalents ranged from 0–29 with a mean value of 16.96. The ring bivalent ranges from 0–11 with a mean value of 4.76 and rod bivalents ranged from 0–24 with a mean value of 12.20. Trivalents ranges from 0–21 with a mean value of 8.52. Quadrivalent associations ranged from 0 to 3 with a mean value of 0.56. The total number of univalent was 33. The total number of chiasmata observed was 1365 with a mean value of 48.75 (Table 2). The total number of terminalized chiasmata was 1003 and unterminalized was 362 and yielding a terminalization coefficient of 0.74.

A low frequency of multivalent as compared to bivalent associations was recorded in all the species (Table 3). In group I, the highest percentage of bivalents was recorded in *C. mangga* (94.02%) and lowest in *C. haritha* (64.12%) and the lowest multivalent association was recorded in *C. haritha* (2.11%) and highest in *C. montana* (5.14%). In group II, the highest frequency of bivalents was recorded in *C. sylvatica* (61.99%) and the lowest in *C. longa* (44.31%).

Discussion

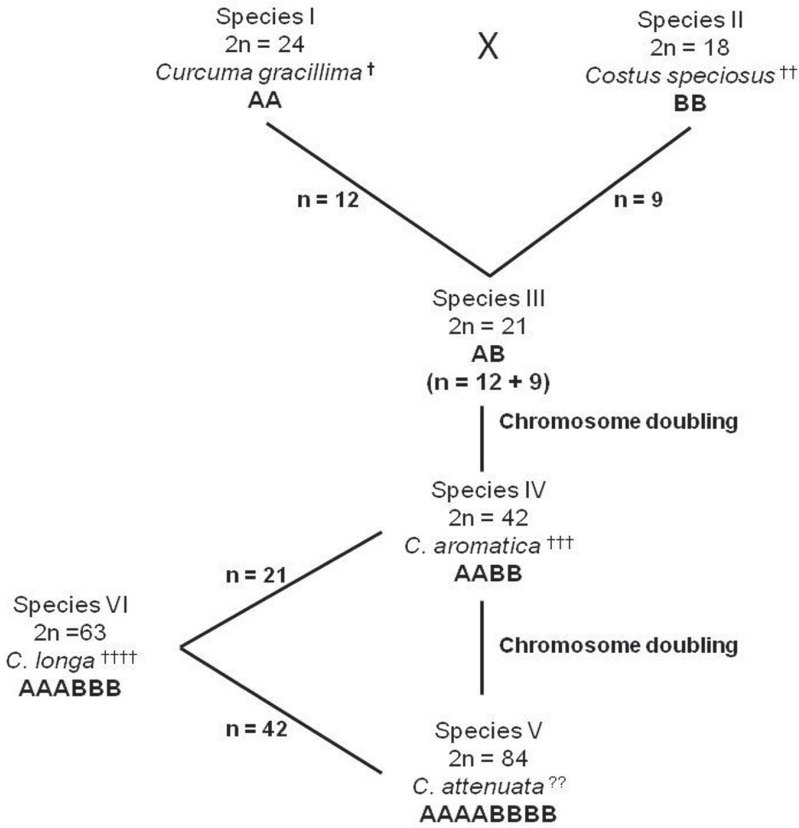
In the present study, seven species of *Curcuma* showed varying degree of chromosome association(s) viz. bivalents, multivalents and univalents. Group I species showed the prevalence of bivalent associations besides univalents and occasional quadrivalents with a near- normal meiotic behaviour. On the other hand Group II species as expected showed trivalent associations besides bivalents, univalents and quadrivalents. Similar observations were also reported by Ramachandran 1961, Nambiar 1979 and Puangpairote et al. 2016 in *C. aromatica, C. decipens, C. longa, C. comosa* and *C. latifolia*. It is interesting to note that univalents in *C. montana* lie in close proximity to each other at diplotene suggesting a residual attraction between homologues and their recent separation (Ghosh et al. 2016). However, in the remaining six species, the occurrence

of univalents cannot be deciphered whether it is a consequence of synaptic variation or precocious separation of the chromosomes.

The present study strongly support that *Curcuma* is an allopolyploid complex which is evident from the low frequency of multivalent associations and in view of the fact that chromosome associations at the first meiotic division are the usual source of information concerning the type of polyploidy in a given plant (Swaminathan 1953). Allopolyploidization mechanisms involving interspecific and intergeneric hybridization, followed by chromosome doubling for obtaining a stable allopolyploid lineage, plays a pivotal role in the plant evolution (Stebbins 1971, Feldman and Levy 2005, Ozkan and Feldman 2009, De Strome and Mason 2014). Allopolyploids are characterized by a diploid-like meiotic behaviour. Male meiotic events in *Curcuma* species clearly signify that species differentiation is helped by polyploid events and the resultant products are yet to be stabilized in nature.

Members of the zingiber family viz. *Zingiber* and *Mantisia* exhibit varying degree of meiotic irregularities have contributed to reduce fertility and poor seed set (Ramachandran 1969, Sharma et al. 2012). This might be the probable reason for vegetative propagation by means of bulbils and rhizomes (Puangpairote et al. 2016). Likewise, *Curcuma* species have also adopted vegetative mode of propagation which apparently help to overcome meiotic disturbances. Furthermore, polyploidy has offered a strong evolutionary advantage to adapt to a wide range of ecological niche and better survivability than their diploid counterpart (Stebbins 1971, Grant 1971, Feldman and Levy 2005). Several studies have reported that *Curcuma* species with 2n = 63 (probable triploids) are geographically widespread (Ramachandran 1961, Škorničková et al. 2007) and have been slightly successful in cultivation, mainly for their productive rhizomes and competitive ability in natural environment (Puangpairote et al. 2016).

From comprehensive male meiotic investigation in seven species of *Curcuma*, we speculate that the speciation in *Curcuma* might have been affected by inter-specific crosses. We hypothesize that *Curcuma* species with 2n = 24 (e.g. *Curcuma gracillima*, etc.) might involved in hybridization events with species of related taxa belonging to the order Zingiberales having 2n = 18 (e.g. *Costus speciosus*) resulting in F_1 progeny with 2n = 21 (Fig. 3). Such hybridization events might be followed by natural and expected chromosome doubling giving rise to amphidiploids with 2n = 42, a somatic number more common in the genus *Curcuma* e.g. *C. aromatica, C. mangga, C. decipens,* etc. In the course of subsequent evolution, these amphidiploid species might have underwent yet another round of chromosome doubling resulting in species derivatives with 2n = 84, a presumed octoploid viz. *Curcuma attenuata*. Few probable triploid species of *Curcuma* such as *C. caesia, C. longa, C. sylvatica,* etc., could be possible due to inter-specific hybridization at heteroploid levels involving amphidiploids (e.g. *C. aromatica, C. comosa, C. mangga,* etc.) and inter-specific octoploid (e.g. *C. attenuata*). Our hypothesis amply gains support from cytogenetical investigation carried out in the present study, wherein male meiotic analysis of amphidiploid species viz. *C. mangga* showed the presence of more bivalents (94.02%) as compared to univalent or any other type of associations. On the other hand, triploid (presumed) species like *C. longa* showed the presence of significant number of trivalents (35.86%), a hallmark



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Synonymous species:

†: *Curcuma, Boesenbergia, Etlingera, Globba, Kaempferia, Roscoea*

††: *Costus* species

†††: *Curcuma aromatica, C. amada, C. comosa, C. coriacea, etc.*

††††: *Curcuma aeruginosa, C. longa, C. caesia, C. elata, etc.*

Figure 3. Proposed scheme for *Curcuma* speciation and diversification.

feature of triploids. However, detail meiotic data from species with 2n = 84, like *C. attenuata* (presumed naturally occurring octoploid) needs to be further investigated for approval of the hypothesis proposed.

Besides the reason for considering *Costus speciosus* as a putative diploid parent is that there is no published literature on chromosome counts with 2n = 18 in any of the species belonging to Zingiberaceae, Hedychieae and Globba, the closely related

tribes of the order Zingiberales. Moreover, Costaceae showed a close relationship with Zingiberaceae and was even previously placed as a subfamily within the family Zingiberaceae and immensely shared broad similarities in inflorescence and floral traits (Specht and Stevenson 2006). Futhermore, x = 21 is too high a basic number to be considered (Škorničková et al. 2007), therefore, we suggest that the genus *Curcuma* has evolved by hybridization of species with different chromosome numbers of 2n = 24 and 18, resulting in a dibasic amphidiploid species which is in complete support of Ramachandran (1961, 1969) and Nambiar (1979) findings with regard to speciation of the genus *Curcuma.*

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Comparative cytogenetics of some marsupial species (Didelphimorphia, Didelphidae) from the Amazon basin

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Abstract

We investigated the karyotype of 18 didelphid species captured at 13 localities in the Brazilian Amazon, after conventional staining, C-banding, Ag-NOR and fluorescent *in situ* hybridization (FISH) using the 18S rDNA probe. Variations were found in the X chromosome, heterochromatin distribution and the 18S rDNA sequence. The main variation observed was in the position of the centromere in the X chromosome of *Caluromys philander* Linnaeus, 1758 and *Marmosa murina* Linnaeus, 1758. For both species, the X chromosome showed a geographical segregation in the pattern of variation between eastern and western Brazil, with a possible contact area in the central Amazon. C-banding on the X chromosome revealed two patterns for the species of *Marmosops* Matschie, 1916, apparently without geographic or specific relationships. The nucleolus organizer region (NOR) of all species was confirmed with the 18S rDNA probe, except on the Y chromosome of *Monodelphis touan* Shaw, 1800. The distribution of this marker varied only in the genus *Marmosa* Gray, 1821 [*M. murina* Thomas, 1905 and *M. demerarae* Thomas, 1905]. Considering that simple NORs are seen as a plesiomorphic character, we conclude that the species *Marmosa* spp. and *Didelphis marsupialis* Linnaeus, 1758 evolved independently to the multiple condition. By increasing the sample, using chromosomal banding, and FISH, we verified that marsupials present intra- and interspecific chromosomal variations, which suggests the occurrence of frequent chromosomal rearrangements in the evolution of this group. This observation contrasts with the chromosomal conservatism expected for didelphids.

Keywords
Marsupials, Amazon basin, C-band, NORs, 18S rDNA, Chromosomal rearrangements

Introduction

In the Americas, subclass Metatheria Huxley, 1880 is represented by the three marsupial orders: Didelphimorphia Gill, 1872, Paucituberculata Ameghino, 1894 and Microbiotheria Ameghino, 1889. The largest of the three American orders is Didelphimorphia, which is represented by the family Didelphidae Gray, 1821, whose species are widely distributed throughout the continent. Didelphidae is the only marsupial group present in Brazil. Together with rodents, they make up an important part of the mammalian fauna of the Amazon region (Voss and Jansa 2009, Wilson and Reeder 2011). Currently, 14 genera and 39 species are recorded in the Amazon basin. Although moderate in terms of species richness, didelphids are abundant in the region (Brandão et al. 2015).

Historically, the first cytogenetic data on American marsupials were recorded by Jordan (1911; cited in Reig et al. 1977), on the spermatogenesis of *Didelphis virginiana* Kerr, 1792. Since then, our knowledge of cytogenetics of American and Australian marsupials has grown significantly. Hayman (1990) reported the karyotype of 178 species of American and Australian marsupials and Svartman (2008) reported 45 karyotypes for American marsupials.

Unlike other mammal orders, such as Rodentia Bowdich, 1821, marsupials show relatively little chromosomal variation (Nagamachi et al. 2015). Chromosomal stability in marsupials was first verified in conventional staining karyotypes that revealed the existence of three main diploid numbers in species from both continents: 14, 18 and 22 chromosomes.

Among all the metatherian families, Macropodidae Gray, 1821 (order Diprotodontia Owen, 1866) is the most diverse in diploid number, varying from 2n=10 to 32. While the American Didelphidae has only the three main diploid numbers, with the most frequent being 2n=14 (Reig et al. 1977, Hayman 1990, Palma and Yates 1996, Carvalho et al. 2002), which has been suggested as the ancestral diploid number of all marsupials (Reig et al. 1977, Westerman et al. 2010). Further comparisons using chromosome banding in American and Australian marsupial species revealed that chromosomal stability is verified not only on the diploid number but also on longitudinal banding patterns that show intense conservation on chromatids (Yonenaga-Yassuda et al. 1982, Rofe and Hayman 1985, Casartelli et al. 1986, Souza et al. 1990, Svartman and Vianna-Morgante 1999).

Limited sampling effort has hampered the estimation of species richness in the Amazon, leaving large gaps in our knowledge of the mammalian fauna (Voss and Emmons 1996, da Silva et al. 2001). Currently, of the 39 species of Amazonian marsupials (Brandão et al. 2015) only 17 have associated cytogenetic data (Nagamachi et al. 2015). However, considering the taxonomic instability of Amazonian marsupials,

this representation might not be accurate, since new phylogenetic studies will probably change the current classification of several taxa. Furthermore, the earlier literature often lacks a connection between the karyotype of putative species and the analyzed specimens, making it difficult to verify *a posteriori* the taxonomic identification attributed to a given karyotype.

The number of taxa analyzed to date is also limited, and existing cytogenetic analyses have been usually restricted only to the diploid and fundamental numbers (Nagamachi et al. 2015). New advances in the taxonomic classification of Amazonian marsupials, complementary techniques of cytogenetic analysis (banding, *in situ* hibridization), and added sampling efforts (more specimens, new localities) are necessary to improve current knowledge on the cytogenetics of these animals.

In this study, we analyze the main morphological differences in the sex chromosomes and the C-band pattern of 18 didelphid species from the Brazilian Amazon. In addition, we describe for the first time karyotype for six species (*Monodelphis touan*, *Monodelphis* aff. *adusta*, *Monodelphis* sp., *Marmosops impavidus*, *Marmosops bishopi* and *Marmosops pinheiroi*) and discuss these patterns in a broader geographical context, including other regions of Brazil and South America.

Material and methods

We cytogenetically analyzed 111 individuals in 18 species and 8 didelphid genera, collected in 13 localities in the Amazon (Table 1 and Figure 1). Scientific collecting permits were obtained from the Brazilian Institute of the Environment and Renewable Natural Resources (Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis – IBAMA), according to SISBIO license numbers: 02005.000642/03-11 (IBAMA); 02000.002336/2003-93 (IBAMA); 02005.002672/04 (IBAMA); 37585-5 (SISBIO); 37592-4 (SISBIO). The specimens were deposited at the Mammals Collection of the National Institute of Amazonian Research (INPA), Manaus, Brazil. Specimens are indicated by species, sampling sites, genus and collector number, followed by INPA collection number (in parentheses) when available,and their field codes are listed bellow. Karyotyped specimens at the figures: Figure 2: a) *Marmosa demerarae* (RNL 46, boxes: MCA 27); b) *Metachirus nudicaudatus* (SISTAP-M-302; boxes: SISSIS-M-64); c) *Gracilinanus emiliae* (SISTAP-M-243); d) *Marmosa murina* (RNL 69, boxes: CEF 18); e) *Caluromys philander* (SISTAP-M-244, boxes: CAN 34, SISTAP-M-305); f) *Caluromys lanatus* (CTGA-M-701); ; g) *Marmosops pinheiroi* (INPA 5377, boxes: EE 192) (SISTAP-M-278, boxes: EE107, INPA 5408);:. Figure 3: a) *Glironia venusta* (BAC 80); b) *Monodelphis* aff. *adusta* (INPA 5388); c) *Monodelphis touan* (INPA 5404); d) *Monodelphis* sp. (CAN 44); e) *Didelphis marsupialis* (EE 249, boxes: EE174)."

All voucher specimens: *Glironia venusta* Thomas, 1912: (BAC 80) – *Caluromys philander* Linnaeus, 1758: Tapajós River (male: SISTAP-M-297; SISTAP-M-305; SISTAP-M-318; SISTAP-M-382; female: SISTAP-M-244); Trombetas River (female:

Table 1. Didelphid species and their respective localities. Species analyzed in the current study were collected at localities 1 to 13, with number of individuals of males (M) and females (F) indicated. Geographic references for the current project were collected in a decimal degree projection using the WGS 84 reference. For literature data we insert converted geographical references where available. Localities with coordinates are presented only the first time they are cited in the table.

Species	Locality	Locality Number	Coordinates†	M	F	Total	Reference
Caluromys philander	Trombetas River, Pará State	10	1.48163888889°S, 56.4573333333°W	9	5	14	Present work
	Tapajós River, Pará State	11	3.35486111111°S, 55.2031666667°W	1	1	2	Present work
	Purus River, Amazonas State	4	4.98066666667°S, 62.9770000000°W		1	1	Present work
	Manaus, Amazonas State	6	3.100548°S, 59.974595°W		1	1	Present work
	Aragua, Venezuela	14	–				Reig et al. 1977
	Manaus, Amazonas State	15	3.13333333333°S, 59.9500000000°W				Souza et al. 2013
	Jari, River, Pará State, Brazil	12	0.7000000000°S, 52.6666666667°W		1	1	Souza et al. 2013
	Pernambuco state	16	–				Souza et al. 1990
	São Paulo state	17					Pereira et al. 2008
Caluromys lanatus	Japurá River, Amazonas State	1	1.84341666667°S, 69.0264722222°W		1		Present work
	Iquitos, Peru	–	–				Hayman and Martin 1974
	Manaus, Amazonas State	–	–				Casartelli et al. 1986
	Rondônia, Brasil	13	–				Souza et al. 1990
Marmosa demerarae	Aripuanã River, Amazonas State	7	6.00000000000°S, 60.1666666667°W	4	4	8	Present work
	Manaus, Amazonas State	6	3.13333333333°S, 59.9500000000°W	7	11	18	Present work
	Cuieiras River, Amazonas State	5	2.70708611111°S, 60.3738388889°W	4	2	6	Present work
	Purus River, Amazonas State	4	0.57725000000°S, 64.8976944444°W	3	4	7	Present work
	Negro River, Amazonas State	3	0.57725000000°S, 64.8976944444°W	1	5	7	Present work
	Tapajós River, Pará State	11	3.35486111111°S, 55.2031666667°W	3	5	9	Present work
	Trombetas River, Pará State	10	1.48163888889°S, 56.4573333333°W	9	5	14	Present work
	Jari River, Pará State	12	0.7000000000°S, 52.6666666667°W	9	2	11	Present work
	Juruá River, Amazonas State	2	3.64151111111°S, 66.1006916667°W		1	1	Present work
	Jatapú River, Amazonas State	9	2.017940°S, 58.203228°W		1	1	Present work
	Jari River, Pará State	12	0.7000000000°S, 52.6666666667°W	1		1	Present work
	Uatumã River, Amazonas State	8	1.84998888889°S, 59.4402000000°W	5	3	9	Present work
	Trombetas River, Pará sate	10	1.48163888889°S, 56.4573333333°W		1	1	Present work
	Negro River, Amazonas State	3	0.57725000000°S, 64.8976944444°W	1	1	2	Present work
	Juruá River	2	3.64151111111°S, 66.1006916667°W		1	1	Present work

Species	Locality	Locality Number	Coordinates†	M	F	Total	Reference
Marmosa murina	Purus River, Amazonas State	4	0.57725000000°S, 64.8976944444°W	2		2	Present work
	Pernambuco State	16	–				Souza et al. 1990
	Villa Vivencio, Colombia	18	–				Hayman and Martin 1974
	Bolivar, Venezuela	19	–				Reig et al. 1977
	Tartarugalzinho, Amapá State	21					Carvalho et al. 2002
	Loreto, Peru	20	–				Reig et al. 1977
	Vila Rica, Mata Grosso State	22	10°01'S, 51°07'W				Pagnozzi et al. 2002
	UHE Peixe Angical, Tocantins State	23	12°01'30"S, 48°32'21"W				Pereira et al. 2008
	Porto Nacional, Tocantins state	24	10°42'S, 48°25'W				Lima 2004
	Uruaçú, Goiás state	25	14°31'S, 49°08'W				Carvalho et al. 2002
	Colinas do Sul, Goiás state	26	14°09'S, 48°04'W				Carvalho et al. 2002
	UHE Corumbá IV Luziania, Goiás state	27	16°15'09"S, 47°57'01"W				Carvalho et al. 2002
	Pacoti, Ceará state	28	4°13'S, 38°55'W				Pagnozzi et al. 2002
	Reserva Biológica Duas Bocas, Espírito Santo state	29	20°16'S, 40°28'W				Paresque et al. 2004
Gracilinanus emiliae	Tapajós River, Pará state	11	35486111111°S, 55.2031666667°W	3	1	4	Present work
	Serra da Mesa, Colinas do Sul, Goiás state	26	14°09'S 48°04'W				Carvalho et al. 2002
	UHE Corumbá IV, Luziania,	27	16°15'09"S, 47°57'01"W				Pereira et al. 2008
Metachirus nudicaudatus	Trombetas River, Pará state	10	1.48163888889°S, 56.4573333333°W		1	1	Present work
	Jari River, Pará state	12	0.7000000000°S, 52.6666666667°W	1		1	Present work
	Cuieiras River, Amazonas state	5	2.70708611111°S, 60.3738388889°W	1		1	Present work
	Juruá River, Amazonas state	2	3.64151111111°S, 66.1006916667°W		1	1	Present work
	Tapajós River, Pará state	11	3.5486111111°S, 55.2031666667°W	2	2	4	Present work
Glironia venusta	Porto Velho, Rondônia State	13	8.87416666667°S, 64.0077777778°W		1	1	Present work
Monodelphis touan	Jari River, Pará state	12	0.7000000000°S, 52.6666666667°W	3		3	Present work
Monodelphis sp.	Purus River, Amazonas state	4	0.57725000000°S, 64.8976944444°W		1	1	Present work
Monodelphis aff. *adusta*	Aripuanã River, Amazonas state	7	6.00000000000°S, 60.1666666667°W	1		1	Present work
Monodelphis emiliae	Aripuanã River, Amazonas state	7	6.00000000000°S, 60.1666666667°W		1	1	Present work
	Juruá River, Acre state		8°40'S 72°47'W				Patton et al. 2000
Monodelphis brevicaudata	Negro River state	3	0.57725000000°S, 64.8976944444°W	1		1	Present work
Marmosops bishop	Aripuanã River, Amazonas state	7	6.00000000000°S, 60.1666666667°W	5	6	11	Present work
	Purus River, Amazonas state	4	0.57725000000°S, 64.8976944444°W	2	1	3	Present work
	Negro River, Amazonas state	3	0.57725000000°S, 64.8976944444°W	1		1	Present work

Species	Locality	Locality Number	Coordinates†	M	F	Total	Reference
Marmosops pinheiroi	Tapajós River, Pará state	11	3.5486111111°S, 55.2031666667°W	4	2	6	Present work
Marmosops parvidens	Trombetas River, Pará state	10	1.48163888889°S, 56.4573333333°W	8	1	9	Present work
	Cuieiras River, Amazonas state	5	2.70708611111°S, 60.3738388889°W	3	2	5	Present work
	Jari River, Pará state	12	0.7000000000°S, 52.6666666667°W		2	2	Present work
	Jatapú River, Amazonas state	9	2.017940°S, 58.203228°W	4	3	7	Present work
	La Paz, Bolívia	–	–				Palma and Yates 1996
	Serra da Mesa, Colinas do Sul, Goiás state	26	14°09'S, 48°04'W				Carvalho et al. 2002
	Apiacás, Mato Grosso state		9°34'S, 57°23'W				Pagnozzi et al. 2002
Marmosops impavidus	Juruá River, Amazonas state	2	3.64151111111°S, 66.1006916667°W	2	1	3	Present work
Marmosops pakaraimae	Japurá River, Amazonas state	1	1.84341666667°S, 69.0264722222°W		1	3	Present work
Didelphis marsupialis	Tapajós River, Pará state	11	3.5486111111°S, 55.2031666667°W	1	3	4	Present work
	Trombetas River, Pará state	10	1.48163888889°S, 56.4573333333°W	1	2	3	Present work
	Manaus, Amazonas state	6	3.13333333333°S, 59.9500000000°W	8	4	12	Present work
	Uatumã River, Amazonas stateM	9	2.017940°S, 58.203228°W	1	1	2	Present work
	Cuieiras River, Amazonas state	5	2.70708611111°S, 60.3738388889°W	2	2	4	Present work

CTGA-M-652); Purus River (female: CAN 34); Manaus (female: MSN 01); (female: BAC 102) – *Caluromys lanatus* Olfers, 1818: Japurá River (female: CTGA-M-701) – *Marmosops* sp. Matschie, 1916: Aripuanã River (female: MCA 3; MCA 7; MCA 8; MCA 26; MCA 31; MCA 35; male: MCA 4; MCA 16; MCA 38; MCA 39); Jari River (female: TAG 3459; RNL 70); Juruá River (male: EE 107; EE 139; female: EE135); Cuieiras River (female: EE 198; EE 211; male: EE 192; EE 201; EE216) – *Marmosops bishopi* Pine, 1981: Negro River (male: SISIS-M-127); Purus River (male: SISPUR-M-135; SISPUR-M-157; SISPUR-M-160; SISPUR-M-164; SISPUR-M-135; CAN 30; CAN 51; female: CAN 48) – *Marmosops pinheiroi* Pine, 1981: Tapajós River (male: SISTAP-M-237; SISTAP-M-278; female: SISTAP-M-268; SISTAP-M-277) – *Marmosops parvidens* Tate, 1931: Trombetas River (male: CTGA-M-501; CTGA-M-516; CTGA-M-531; CTGA-M-532; CTGA-M-551; CTGA-M-555; CTGA-M-581; CTGA-M-600; female: CTGA-M-533) – *Marmosops impavidus* Tschudi, 1845: Purus River (male: SISPUR-M-149) – *Marmosops* cf. *pakaraimae* Voss, Lim, Díaz-Nieto et Jansa 2013: Japurá River (male: SISJAP-M-705) – *Marmosa murina* Linnaeus, 1758: Jari River (male: RNL 45); Uatumã River (male: CEF 4; CEF 8; CEF 18; CEF 27; CEF 28; CEF 32; female: CEF 16; CEF 34; CTGA-M-8; CTGA-M-22; CTGA-M-41;), Negro River (male: SISIS-M-57; SISIS-M-63); Trombetas River (female: CTGA-M—519); Purus River (male: CAN 43); Japurá River (male: CTGA-M-708)

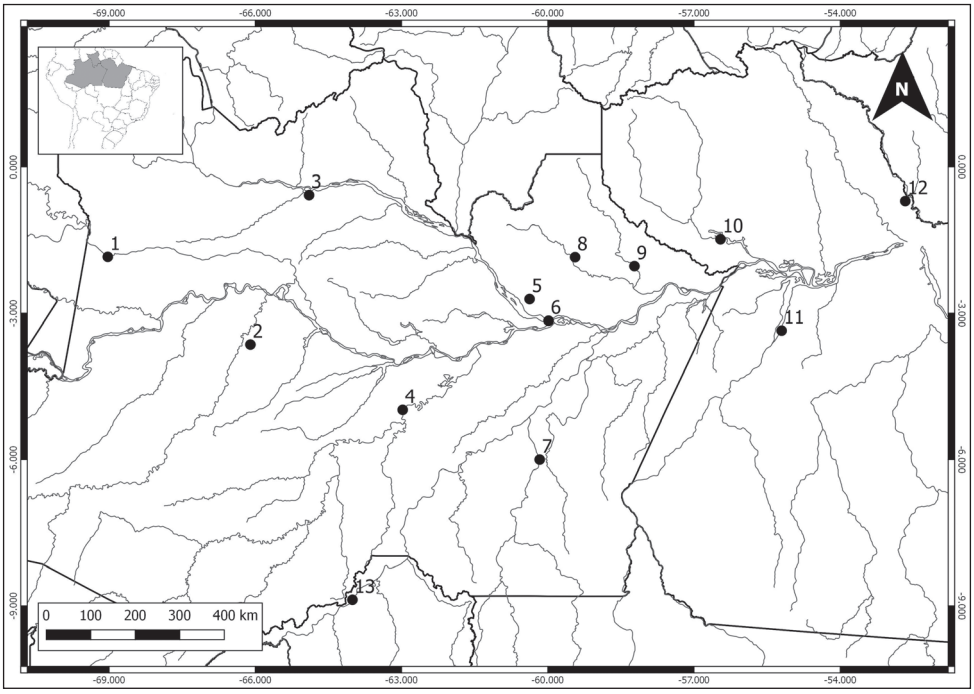

Figure 1. Sampling sites plotted on the Amazon basin map, Amazonas State: **1** Japurá River, Japurá city **2** Juruá River, Juruá city **3** Negro River, Santa Isabel do Rio Negro city **4** Purus River, Tapauá city **5** Cuieiras River, Manaus city **6** Manaus city, urban área: Federal University of Amazonas's campus (UFAM) and Isaac Sabá Oil Refinery) **7** Aripuanã River, Novo Aripuanã city **8** Uatumã River, Presidente Figueiredo city **9** Jatapú River, São Sebastião do Uatumã city; Pará State: **10** Trombetas River, Oriximiná city **11** Tapajós River, Aveiro and Santarém cities **12** Jari River, Almeirim city; Rondônia State: **13** Madeira River, Porto Velho city. Geographic coordinates at the Table 1.

– *Marmosa murina* Linnaeus, 1758: Aripuanã River (female: MCA12, Japurá River (male: SISJAP-M-764)- *Gracilinanus emiliae* Thomas, 1909: Tapajós River: (male: SISTAP- M-245; SISTAP- M-343; SISTAP- M-344; SISTAP- M-345) – *Marmosa demerarae* Thomas, 1905: Aripuanã River (female: MCA 27; MCA 36; MCA 58; MCA 65; male: MCA 21; MCA 59); Jari River (female: RNL 31; RNL 48; male: RNL 30; MCA 32; MCA 46; MCA 49; MCA 58; MCA 61; MCA 64; MCA 66; MCA 67) Juruá River (female: EE136; male: EE 143); Manaus (female: EE 149: EE 150; EE 151; EE 154; EE 158; EE 159; EE 169; EE 222; EE 228; 229; EE 234; male: EE 157; EE 167; EE 170; EE 176; EE 189; EE 194; EE 196; EE 202; EE 215; EE 220; EE 235); Cuieiras River (female: EE 193; EE 219); Tapajós River (female: SISTAP-M-229; SISTAP-M-241; SISTAP-M-321; SISTAP-M-333; SISTAP-M-369; male: SISTAP-M-267; SISTAP-M-279; SISTAP-M-322); Trombetas River (female: CTGA-M-579; CTGA-M-590; CTGA-M-622; CTGA-M-667; CTGA-M-672; male: CTGA-M-535; CTGA-M-539; CTGA-M-557; CTGA-M-558; CTGA-M-572; CTGA-M-573; CTGA-M-578; CTGA-M-580; CTGA-M-613); Negro River (female:

SISIS-M-85; SISIS-M-110; SISIS-M-117; SISIS-M-128; male SISIS-M- 86); Purus River (female: SISPUR-M-145; CAN 25; CAN 31; CAN 50: male: SISPUR-M-144; SISPUR-M-147; SISPUR-M-148) – *Monodelphis* aff. *adusta* Thomas, 1897: Madeira River (male: MCA 15) – *Monodelphis touan*: Jari River (male: TAG 2731; RNL 68) – *Monodelphis* sp. Burnett, 1830: Purus River: (male: CAN 44) – *Monodelphis emiliae* Thomas, 1912: Aripuanã River (female: MCA 31) – *Metachirus nudicaudatus* Geoffroy et Saint-Hilaire, 1803: Jari: River (RNL 47); Cuieiras River: (female: EE 200); Tapajós River (female: SISTAP-M-230; SISTAP-M-230; male: SISTAP-M-251; SISTAP-M-269); Trombetas River: (female: CTGA-M-655); Jatapú River: (female: CTGA-M-52; CTGA-M-58); Negro River: (female: SISIS-M-64; SISIS-M-78; male: SISIS-M-84; SISIS-M-116); Purus River: (male: CAN 33) – *Didelphis marsupialis* Linnaeus 1758: Jari River: (female: RNL 44; RNL 53; RNL 59; male: RNL 52; RNL 55; RNL 62; RNL 63); Manaus: (female EE 174; EE 197; EE 204; EE 224; EE 227; EE 246; EE 250; EE 155; EE 155; EE 173; EE 183; EE 190; EE 203; EE 205; EE 206; EE 223; EE 232; EE 233; EE 237; EE 247;EE 248; EE249; EE 190); Uatumã River (female: CEF 5; male: CEF 13); Trombetas River (female: CTGA-M-594; CTGA-M-606; male: CTGA-M-607); Purus River (male: SISPUR-M-185); Negro River (male: SISIS-M-73):Tapajós River (female: SISTAP-M-324; SISTAP-M-346; SISTAP–M-347;male: SISTAP-M-243); Japurá River: (male: CTGA-M-732).

The metaphases were obtained from bone marrow by *in vivo* method according to Ford and Harmerton (1956). Each animal received 1mL/100g weight of a 0,0125% colchicine solution for 30 minutes, the cells were exposed for 20 minutes to a 0,075M KCl solution, fixed 3:1 in methanol and acetic acid and stored at -20 °C. The C-band and Nucleolus Organizing Regions (NORs) patterns were determined according to the techniques described by Sumner (1972), and Howell and Black (1980), respectively. Chromosome pairing considered morphology in decreasing order of size and the chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) according to the ratio of chromosome arms and the position of the centromere, according to Patton (1967). 18S rDNA sequences were mapped by fluorescence *in situ* hybridization (FISH) according to Pinkel et al. (1986), whose probe was obtained by polymerase chain reaction (PCR) using the following primers designed by Gross et al. (2010): 18SF (5'-CCG CTT TGG TGA CTC TTG AT-3') e 18SR (5'-CCG AGG ACC TCA CTA AAC CA-3') and labeled with digoxigenin (DIG-Nick translation, ROCHE) or Biotin (Bio-Nick translation, ROCHE), following manufacturer's instructions.

Results

Among the 18 species analyzed, 11 showed 2n=14; six 2n=18 and one 2n=22 chromosomes (Table 1).

In the species with 2n=14, we observed a very similar structure among the autosomes. These karyotypes include six autosome pairs (Fig. 2), three large submetacentric

pairs, one metacentric pair and two small pairs that varied in morphology in the different species, resulting in differences in the chromosomal formulas and fundamental numbers (FNa). FNa=20, with formula 2m+6sm+4a+XX/XY, was recorded in *Marmosa demerarae* (Fig. 2a-I) and *Metachirus nudicaudatus* Geoffroy an Saint-Hilaire, 1803 (Fig. 2b-I). FNa=22, with formula 2m+6sm+2st+2a+XX/XY, was present in *Gracilinanus emiliae* Thomas, 1909 (Fig.2c-I), *Marmosa murina* (Fig. 2d-I), *Caluromys philander* (Fig. 2e-I) and *Caluromys lanatus* Olfers, 1818 (Fig. 2f-I). FNa=24, with formula 6m+6sm+XX/XY, was recorded in species of the genus *Marmosops* including *M. bishopi* (Pine, 1981), *M. pinheiroi* Pine, 1981, *M. parvidens* Tate, 1931, *M. impavidus* Tschudi, 1845, and *M.* cf. *pakaraimae* Voss, Lim, Díaz-Nieto et Jansa 2013. The five species of *Marmosops* presented similar karyotypic characteristics (Fig. 2g-I – only *M. pinheiroi* shown).

We observed three different morphologies for X chromosome: metacentric in *G. emiliae* and *Marmosops* spp. (Fig. 2c-I and 2g-I); submetacentric in the only female of *C. lanatus* (Fig. 2f-I); and acrocentric in *M. demerarae* and *M. nudicaudatus* (Fig. 2a-I and 2b-I). In *Caluromys philander* and *Marmosa murina*, we observed an intraspecific variation in the structure of the X chromosome, acrocentric and submetacentric, both in specimens from the same and different localities (Fig. 2e-I and 2d-I).

The bare-tailed woolly opossum (*C. philander*) has X chromosome either acrocentric or submetacentric, with females being either homozygous or heterozygous carriers of the heteromorphic X (Fig. 4). In the murine mouse opossum (*Marmosa murina*), the metacentric or submetacentric X was found in individuals throughout the Brazilian Amazon, except in the Purus River (Fig. 5, locality 4); it was also found in individuals from two localities in central Brazil (Fig. 5, localities 25 and 26). These are situated at the southern limits of the distribution of *M. murina* and both, the submetacentric X and the acrocentric X, are sympatric at locality 26. Furthermore, in the northern Amazon in Colombia, Venezuela and Peru, the X chromosome is metacentric (Fig. 5, localities 18, 19 and 20) (Reig et al. 1977, Carvalho et al. 2002). The acrocentric X was found in the Purus River (Fig. 5, locality 4), and in central, southeastern and northeastern Brazil (Fig. 5, localities 16 and 22-28) (Souza et al. 1990, Palma and Yates 1996, Carvalho et al. 2002).

The Y chromosome was acrocentric in *G. emiliae*, *Marmosops* spp., *M. demerarae* and *M. nudicaudatus* (Fig. 2c, g, a, b), and dot-like in *C. philander* and *M. murina* (Fig. 2e, d).

Among the species with 2n=18 chromosomes, FNa=20 was recorded in *Glironia venusta* Thomas, 1912, with formula 2m+2sm+2st+10a+XX/XY (Fig. 3a-I), FNa=30 was recorded in four species of the genus *Monodelphis* Burnett, 1830: *M.* aff. *adusta* Thomas, 1897 (Fig. 3b-I), *M. touan* (Fig. 3c-I), *M. emiliae* Thomas, 1912 (Fig. 3d-I), and *M. brevicaudata* Erxleben, 1777 (Fig. 3e-I) with formula 2m+2sm+8st+2a+XX/XY, and FNa=32 in *Monodelphis* sp. (Fig. 3f-I), with formula 2m+2sm+10st+2a+XX/XY. We observed two X chromosomes morphologies: acrocentric in *M.* aff. *adusta*, *M. touan* and *M. brevicaudata* (Fig. 3b, c, e), and submetacentric in *Monodelphis emiliae* and *Monodelphis* sp. (Fig. 3d, f). The Y chromosome was acrocentric in *M. touan* and *Monodelphis* sp., and dot-like in *M.* aff. *adusta* and *M. brevicaudata*.

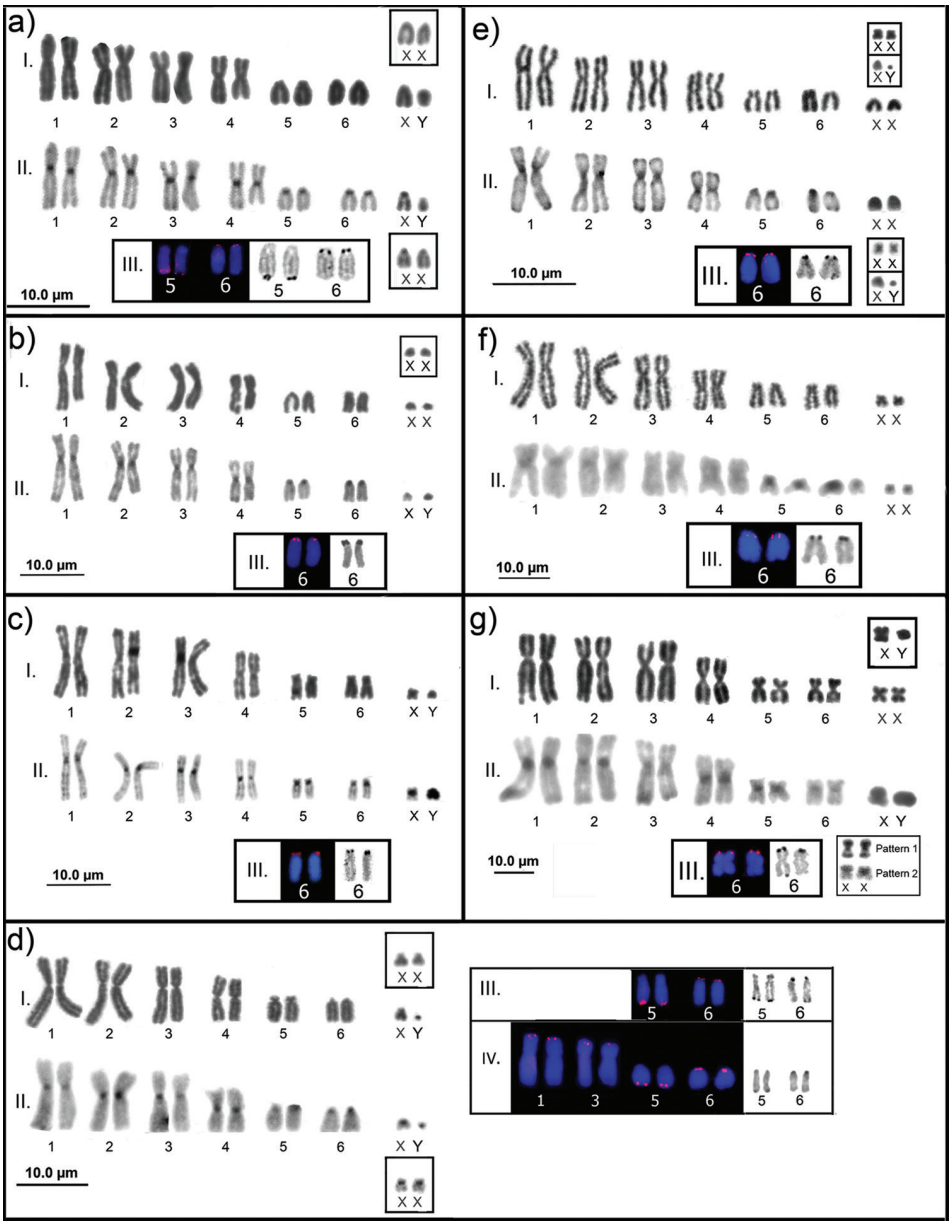

Figure 2. Karyotypes under conventional staining (I), C-band (II), 18S rDNA and Ag-NOR (III), sex chromosomes in the boxes: **a** *Marmosa demerarae* **b** *Metachirus nudicaudatus* **c** *Gracilinanus emiliae* **d** *Marmosa murina*, (IV) variations on the 18S sites found in the individuals from Purus River, Tapauá city, Amazonas State **e** *Caluromys philander* **f** *Caluromys lanatus* **g** *Marmosops pinheiroi*.

Didelphis marsupialis was the only species that presented 2n=22 chromosomes and FNa=20, with formula 20a+XX/XY (Fig. 3g-I), with acrocentric X and Y.

The position of the heterochromatin on the 2n=14 species was centromeric, being conspicuous in *M. demerarae* (Fig. 2a-II), *M. nudicaudatus* (Fig. 2b-II), *G. emiliae*

(Fig. 2c-II), *M. murina* (Fig. 2d-II), and *M. pinheiroi* (Fig 2g-II). *Caluromys philander* and *C. lanatus* exhibited tenuous heterochromatin, with additional telomeric heterochromatin in *C. philander* chromosomes (Fig. 2e-II and 2f-II). The X chromosome in *C. philander* was entirely heterochromatic, except for a distal band in the long arms (Fig. 2e-II); in *M. demerarae* it was also entirely heterochromatic, except for a proximal euchromatic band in the long arms (Fig. 2a-II); in *M. murina* (Fig. 2d-II), *M. nudicaudatus* (Fig. 2b-II) and *G. emiliae* the heterochromatin was centromeric (Fig. 2c-II).

Two C-band patterns were present in the X chromosome for species of *Marmosops*. In pattern 1, X was entirely heterochromatic except for a proximal band in the long arms (Fig. 2g – box); in pattern 2, the heterochromatin was in the short arms and the centromere (Fig. 2g – box). Both patterns were present in *M. parvidens* and *M. bishopi*, while only pattern 1 was observed in *M.* cf. *pakaraimae*, *M. impavidus* and *M. pinheiroi* (Table 2). The Y chromosome was entirely heterochromatic in all species.

In the species with 2n=18 chromosomes, the heterochromatin was centromeric in *G. venusta* (Fig. 3a-II), *M.* aff. *adusta* (Fig. 3b-II), *M. touan* (Fig. 3c-II) and *M. emiliae* (Fig. 3d-II). The Y chromosome was entirely heterochromatic in *M. adusta* (Fig. 3b-II) and *M. touan* (3c-II). It was not possible to determine the C-band pattern in *Monodelphis* sp. and *M. brevicaudata*.

NORs confirmed by FISH using the 18S rDNA probe were present in the short arms of pair 6 in all 2n=14 species and *G. venusta* (2n=18). In *M. demerarae* and *M. murina* sites were also detected in the terminal position of the long arms of pair 5 (Fig. 2, a-III e d-III). In *M. emiliae* (2n=18) the NOR was positioned on the short arms of pair 7 (Fig. 3d-III), and in *M. touan* in the X and Y chromosomes, although no 18S site was detected in Y (Fig. 3c-III). Only *Monodelphis brevicaudata* exhibited multiple NORs (Fig. 3e-III), whose sites were in the terminal region of the long arms of pair 7 and the short arms of X and Y.

In *D. marsupialis*, both the 18S rDNA probe and silver were detected in three chromosome pairs. In two pairs, the sites were located in the terminal region of the long arms, while in one pair they were bitelomeric (Fig. 3g-III). However, in regards to activity, there was a variation of four to eight markings.

Discussion

In the last decade, advances in systematic and taxonomic studies of the family Didelphidae introduced changes in the taxonomy and nomenclature of several of its taxa (Jansa and Voss 2000, Voss and Jansa 2003, Voss and Jansa 2009, Rossi et al. 2010, Gutiérrez et al. 2010). We used the phylogenetic tree of Jansa and Voss (2014) to map the cytogenetic data of the 18 species we have analysed in order to gain an understanding of chromosome evolution in the group. This work represents the most updated phylogeny of the intergeneric relationships of didelphid marsupials, making our interpretation of the cytogenetic data more integrative than a mere consideration of chromosomal data, and more accurate in light of an independently generated phylogenetic hypothesis.

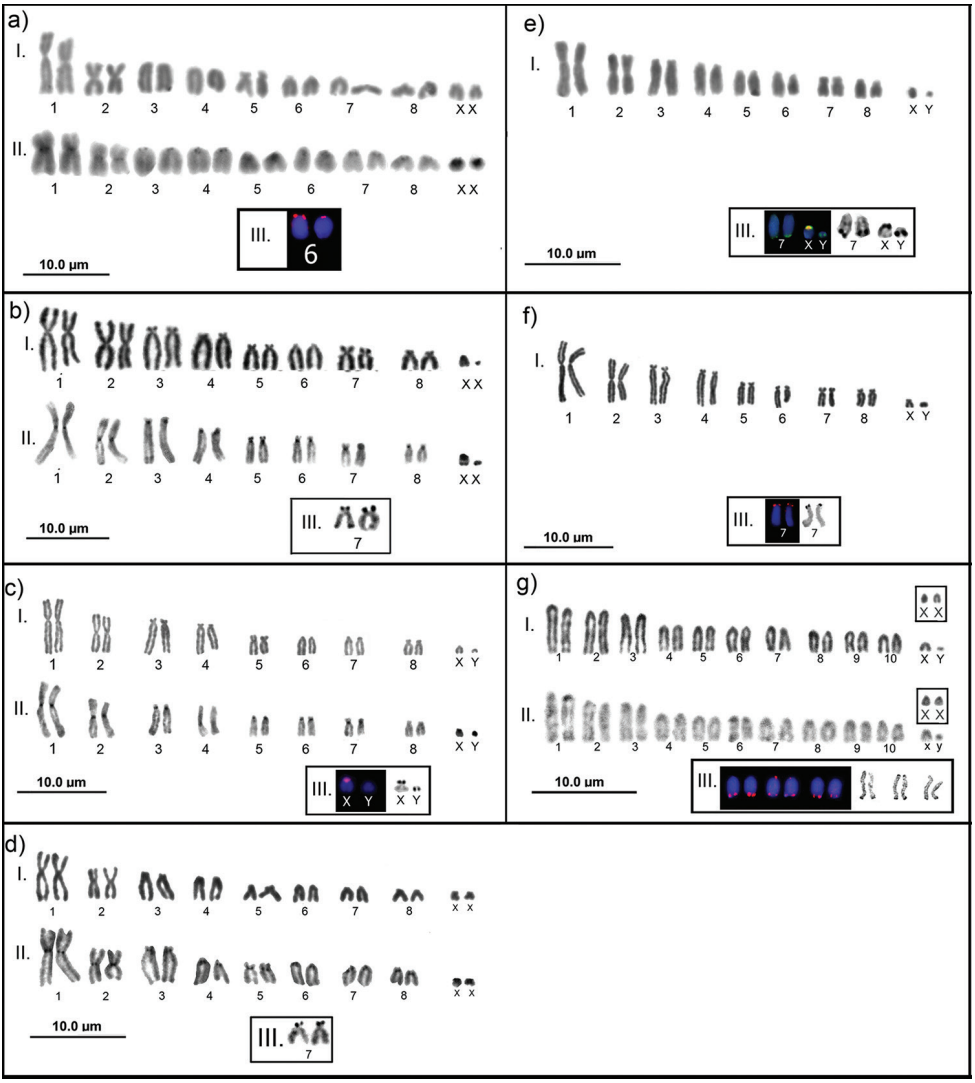

Figure 3. Karyotypes under conventional staining (I), C-band (II), 18S rDNA and Ag-NOR (III), sex chromosomes in the boxes: **a** *Glironia venusta* **b** *Monodelphis* aff. *adusta* **c** *Monodelphis touan* **d** *Monodelphis emiliae* **e** *Monodelphis brevicaudata* **f** *Monodelphis* sp. **g** *Didelphis marsupialis*.

The autosome structure observed here corroborates karyotypic conservation in the diploid number and chromosomal formula (NFa) as previously described in the didelphid species *Didelphis marsupialis, Marmosa demerarae, Metachirus nudicaudatus, Monodelphis touan* (previously named *M. brevicaudata*), *Monodelphis* aff. *adusta* (previously named as *M.* cf. *emiliae*) and for species of *Marmosops* (Reig et al. 1977, Yonenaga-Yassuda et al. 1982, Casartelli et al. 1986, Hayman 1990, Souza et al. 1990, Palma and Yates 1996, Svartman and Vianna-Morgante 1998, 1999, Carvalho et al. 2002).

Although didelphids are generally considered to have a conserved karyotype, by comparing the karyotypes among different genera, it was possible to associate them with certain species due to the presence of diagnostic characters. For example, *M. demerarae* and *M. murina* differ in their FNa, morphology, and sex chromosome size. In species of the genus *Monodelphis*, morphological variation in chromosomes was restricted to pair 6, which grants an FNa varying between 30 (as observed in *M.* aff. *adusta*, *M. touan* and, *M. brevicaudata*) and 32 arms (*Monodelphis* sp.). However, the same does not occur for the genus *Marmosops,* in which the five species analysed, present a very similar chromosome macrostructure.

The genus *Marmosa* has a complex taxonomy and recently underwent great taxonomic changes, with all species of *Micoureus,* formerly treated as a separate genus, now considered as a subgenus of *Marmosa*. Considering the taxonomic instability in Didelphidae, with individuals being reclassified, and some complex of species being divided into two or more valid taxa, even purportedly karyotyped species may in fact have their karyotypes still unknown. Thus, our knowledge as to how many and which species among didelphids were karyotyped remains unstable. A revision of the literature for species with reported karyotypes is required.

X chromosome variations

Souza et al. (2013) observed different forms of the X chromosome in *Caluromys philander*, and our data contribute to show their wide geographic distributions. The acrocentric X are found in northeastern and southeastern Brazil (Fig. 4, localities 16 and 17), as well as in central (Fig. 4, locality 6) and eastern Amazon (Fig. 4, localities 10, 11 and 12). While submetacentric form is located in Venezuela (Fig. 4, locality 14) and areas in the western, central and eastern Amazon (Fig. 4, localities 4, 6, 12 and 15) (Reig et al. 1977, Svartman and Vianna-Morgante 1999, Pereira et al. 2008). Interestingly, both homozygote and heterozygote females were recorded in central Amazonia near Manaus (Fig. 4, locality 6). It is not clear how often this condition is found in natural populations. Indeed, so far, the few heterozygous records for X might be related to the low number of captured and cytogenetically analyzed individuals.

Apparently, there is a likely geographical structure in the distribution of the morphological forms of the X chromosome in *Marmosa murina*, with the metacentric X so far found in the northern and western parts of its distribution, the submetacentric X prevailing in the Amazon basin of Brazil and the acrocentric forms prevailing in the other known localities in central and eastern Brazil (Fig. 5). According to Brito et al (2015), this species is currently under revision and is likely to be split into three species. It remains to be seen if there will be a correspondence between those species and the karyotypic forms depicted here.

Among the Amazonian marsupials analyzed here, the variation in centromere position and heterochromatin patterns of the X chromosome is noteworthy. Souza et al. (2013) suggested that pericentric inversions in the X chromosome of *Caluromys*

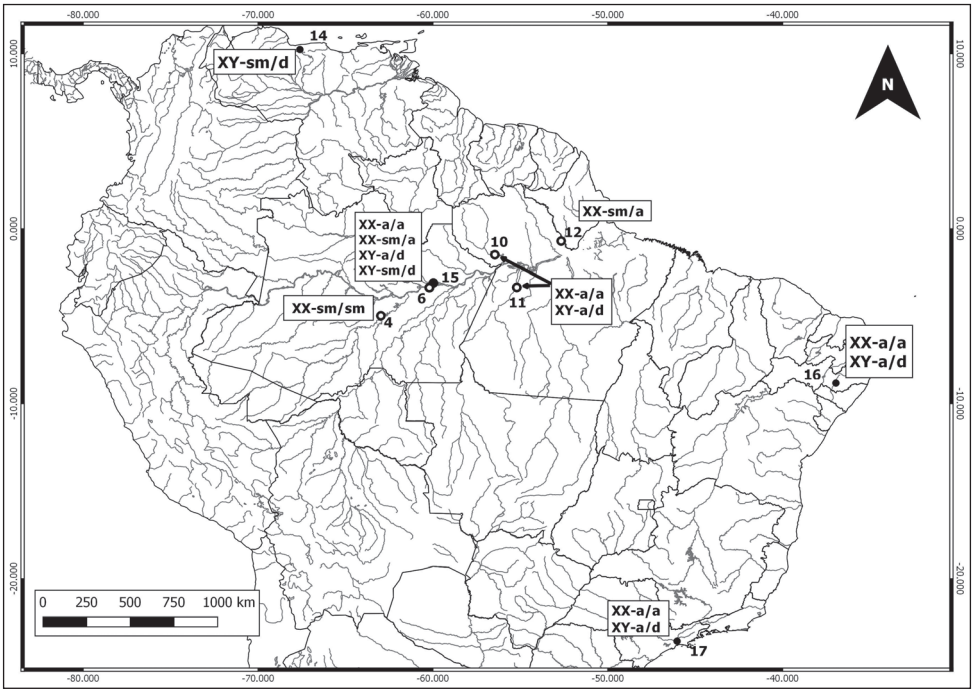

Figure 4. Geographic locations of *Caluromys philander* individuals and its sexual chromosomes morphology data in South America. Literature data represented by empty circles and present work represented by full circles: (● 14) Venezuela, Reig et al. 1977; (○ 4) Purus River; (○ 6) Manaus city, urban área: Federal University of Amazonas's campus (UFAM); (● 15) Manaus REMAN (Isaac Saba Oil Refinery), present work and Souza et al. 2013; (○ 10) Trombetas River; (○ 11) Tapajós River; (○ 12) Jari River, Souza et al. 2013; (● 16) Pernambuco State, Souza et al. 1990; (● 17) São Paulo State, Svartman and Vianna-Morgante 1999 and Pereira et al. 2008. m=metacentric; sm=submetacentric; a=acrocentric; d=dot-like.

philander altered its morphology, and our results support their findings. In contrast, in *M. murina*, the different morphologies (m, sm, and a) of chromosome X might be due to centromeric shift without the presence of rearrangements. Such reorganization was already observed in other mammals and might be related to three main regions of the chromosome: subtelomeric, proximal and in the boundary between heterochromatin and euchromatin (Rocchi et al. 2012, Burrack and Berman 2012).

Heterochromatin distribution

We observed chromosomal conservatism in the heterochromatin pattern in eight didelphid species: (*C. lanatus*, *G. venusta*, *D. marsupialis*, *M. touan*, *M.* aff. *adusta*, *M. emiliae*, *G. emiliae* and *M. nudicaudatus*). *C. philander* presented heterochromatic pattern different from the heterochromatic distribution reported in the literature for this

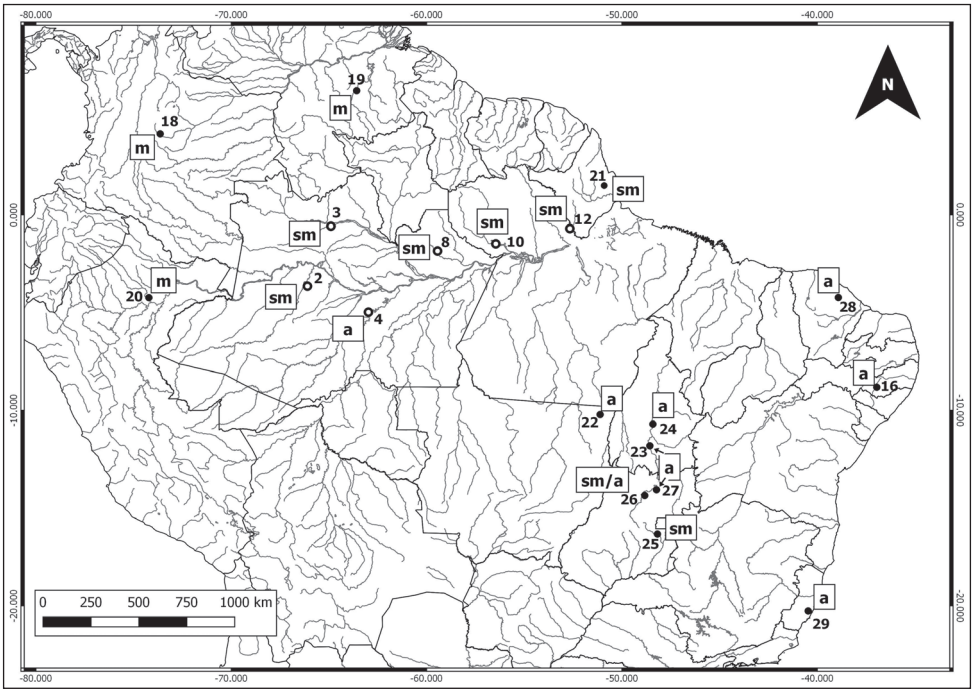

Figure 5. Geographic locations of *Marmosa murina* individuals and its X chromosome morphology data in South America. Literature data represented by empty circles and present work represented by full circles: (● 18) Villa Vivêncio, Colômbia, Hayman and Martin 1974; (● 20) Loreto-Peru, Reig et al. 1977; (● 19) Bolívar, Venezuela, Reig et al. 1977; (○ 3) Negro River; (○ 2) Juruá River; (○ 4) Purus River; (○ 8) Uatumã River; (○ 10) Trombetas River; (○ 12) Jari River; (● 16) Pernambuco State, Souza et al. 1990; (● 21) Tartarugalzinho, Amapá State State, Carvalho et al. 2002; (● 22) Vila Rica Mato Grosso State, Pagnozzi et al. 2002; (● 23) UHE Peixe Angical,TO, Pereira et al. 2008; (● 24) Tocantins State, Lima 2004; (● 25) Uruaçú, Goiás State , Carvalho et al. 2002; (● 26) Colinas do Sul, Goiás state; (● 27) UHE Corumbá IV Luziania, Goiás state Pereira et al. 2008; (● 28) Pacoti, Ceará State, Pagnozzi et al. 2002; (● 29) Espírito Santo State, Paresque et al. 2004. m=metacentric; sm=submetacentric; a=acrocentric.

species (Souza et al. 1990, Souza et al. 2013). In *Marmosops* spp., the C-band patterns of the X chromosome are widespread throughout the Amazon basin, but are found in sympatry in the area between the confluences of the Negro-Purus and the Trombetas-Tapajós Rivers, forming pattern 1 to the west and pattern 2 to the east (Table 2). It remains to be seen if there is a correspondence between these patterns with possible cryptic species to be uncovered by broader molecular systematics and morphological studies of these taxa.

Thus, heterochromatin distribution patterns can serve as a cytotaxonomic character, as well as shedding light on chromosomal evolution and regulation of gene expression. However, our results demonstrate that, except for *Marmosops* spp., the other species under study presented little heterochromatin intraspecific variation, including the X chromosome. Thus, this character alone does not allow for distinguishing among

Table 2. Comparative cytogenetic data of the didelphid species analyzed in the present study and those from the literature. In Locality, numbers indicate sampling sites as in the maps of Figures 1, 4 and 5. Karyotypic data: 2n=diploid number; FNa=autosomal arm number; NOR=Nucleolar Organizer Region; p=short arm; q=long arm. Letters identify the X chromosome morphology: m=metacentric; sm=submetacentric; a=acrocentric; d=dot-like. X chromosome C-Band patterns are identified by A= Centromeric heterochromatin; B= Totally heterochromatic except for a terminal euchromatic band; C= Totally heterochromatic except for an interstitial euchromatic band; D= short arm and centromere totally heterochromatic.

Species	Locality number	2n	FNa	NORs Pair/arm	18S rDNA	X/Y	X chromosome C–band	Source
Caluromys philander	10; 11; 15; 16	14	22	6p	6p	a/d	B	Souza et al. 1990; Souza et al. 2013; Present work
	4; 6; 14; 15; 17	14	22	6p	6p	sm/d	B	São Paulo State, Svartman and Vianna–Morgante 1999, Pereira et al. 2008, Souza et al. 2013, Present work
	12	14	22	6p	6p	sm/a	B	Souza et al. 2013
Caluromys lanatus	1	14	22	6p	6p	sm/–	A.	Present work
Marmosa murina	2; 3; 8; 10; 12; 18; 19; 20; 25; 26	14	22	5q;6p	5q;6p	(m) sm/ d	A	Hayman and Martin 1974, Reig et al. 1977, Pereira et al. 2008, Carvalho et al. 2002, Present work
	16; 22; 25; 24; 26; 27; 28; 29	14	22	5q;6p	5q;6p	a/ d	A	Carvalho et al. 2002, Pagnozzi et al. 2002, Lima 2004, Paresque et al. 2004, Pereira et al. 2008
	4	14	22	5q;6p	1p; 3p; 5q; 6p	a/d	A	Present work
Marmosa demerarae	2; 3; 4; 5; 6; 7; 9; 10; 11; 12 25, 26	14	20	5q; 6p	5q;6p	a/a	C	Carvalho et al. 2002, Present work
	La Paz, Bolívia	14	20	–	–	a/a	–	Palma and Yates 1996
	16	14	24	5q; 6p		a/a		Souza et al. 1990;
	–	14	24	5pq; 6p	5pq; 6p	a/a		Svartman and Vianna Morgante 2003
	Rio Grande do Sul	14	24	5pq; 6p	–	a/a		Carvalho et al. 2002
Marmosops bishopi	4; 7;	14	24	6p		m/a	C; D	Present work
	3	14	24	6p		m/a	C	Present work
Marmosops pinheiroi	11	14	24	6p	6p	m/a	C	Present work
Marmosops parvidens	5; 10; 12	14	24	6p		m/a	C; D	Present work
	9	14	24	6p		m/a	D	Present work
Marmosops impavidus	2	14	24	6p		m/a	C	Present work
Marmosops pakaraimae	1	14	24	6p		m/a	C	Present work
Gracilinanus emiliae	11; 25; 26	14	22	6p	6p	m/a	A	Carvalho et al. 2002, Present work
Metachirus nudicaudatus	2; 5 10; 11; 12	14	20	6p	6p	a/a	A	Present work

Species	Locality number	2n	FNa	NORs Pair/arm	18S rDNA	X/Y	X chromosome C–band	Source
Glironia venusta	13	18	20	6p	6p	a/–	A	Fantin e da Silva 2011, Present work
Monodelphis touan	12	18	28	Xp	Xp	a/a	A	Present work
Monodelphis sp.	4	18	32	7p	7p	sm/a	–	Present work
Monodelphis aff. *adusta*	7	18	30	7p		a/d	A	Present work
Monodelphis emiliae	7, Juruá River, Acre	18	30	7p		sm/–	A	Patton et al. 2000, Present work
Monodelphis brevicaudata	3;	18	30	7q, Xp, Yq	7q, Xp, Yq	sm/a	–	Present work
	Roraima and Pará states	18	30	Xp		a/d		Carvalho et al. 2002
Didelphis marsupialis	5; 6; 9; 10; 11	22	20	5q;7pq;8q	5q;7p-q;8q	a/a	A	Present work

didelphid populations, although heterochromatin distribution may be an effective character for distinguishing between certain species pairs. This is the case for *M. demerarae* and *M. murina*, with the former presenting larger centromeric heterochromatic blocks than the latter, and between *C. philander* and *C. lanatus*, both with 2n=14 and NF=24, but with distinct heterochromatic patterns.

Nucleolus organizer regions (NORs) and their evolution

The NOR in Didelphidae can be simple or multiple. According to Hsu et al. (1975), the single NOR would be an ancestral character in mammals, with subsequent rearrangements leading to multiple NORs in derived groups. The presence of NOR in sex chromosomes also could be considered a derived character since originally it was present in autosomes and ended up in the X chromosome due to rearrangements such as translocation or transposition. The NOR in *Glironia*, *Monodelphis, Caluromys, Gracilinanus*, and *Marmosops* is simple. Thus, these genera have a plesiomorphic condition for this character. Conversely, the species of *Didelphis*, *Marmosa* and *Philander* have the derived condition of multiple NORs (Yonenaga-Yassuda et al. 1982, Svartman and Vianna-Morgante 2003).

According to the literature, in *Monodelphis* there are NOR sites on pair 7 and on the X chromosome of *Monodelphis* aff. *adusta* and *Monodelphis* sp. (Svartman and Vianna-Morgante 1999, Merry et al. 1983, Carvalho et al. 2002). In *M. touan* and *M. brevicaudata* there are simple NORs on the X and Y chromosomes, a condition previously identified in *Monodelphis domestica* Wagner, 1842 (Merry et al. 1983, Pathak et al. 1993). Hsu et al. (1975) reported ribosomal genes in mammal sex chromosomes of the bat species *Carollia castanea*. These authors emphasize that NOR in the X chromosome can generate problems with dosage compensation in mammals.

In the Y chromosome of *M. touan*, FISH did not confirm the marking. This situation was verified in other organisms, where precipitation in the heterochromatic regions took place but could lead to an erroneous interpretation of the distribution of this marker (Schneider et al. 2012). Thus, the marking observed (Fig. 3c III) was not a ribosomal site but a heterochromatic block with silver affinity.

When mapping the NOR character on the phylogenetic tree of Jansa and Voss (2014, fig. 01) (not shown here), we verified that multiple NORs are distributed in two distinct lineages: the first in species of the genus *Marmosa* and the second in species with 22 chromosomes of the genera *Didelphis* and *Philander* Brisson, 1762. The mapping of the simple condition onto the phylogenetic tree depicts a wide distribution for this character, present at the base of the tree (*Caluromys philander, C. lanatus, Glironia venusta*) and in at least one or more species of the remaining major clades (*Gracilinanus emiliae, Marmosops* spp., *Metachirus nudicaudatus, Monodelphis touan, Monodelphis kunsi, and Monodelphis dimidiata*) (Souza et al. 1990, Palma and Yates 1996, Carvalho et al. 2002, Svartman and Vianna-Morgante 2003, Pereira et al. 2008, Souza et al. 2013). This distribution of NOR character on the didelphid phylogeny is thus congruent with the hypothesis advanced by Hsu et al. (1975) that the single NOR is an ancestral state.

When mapping the NOR character on the phylogenetic tree of Pavan et al. (2014) for the genus *Monodelphis*, we verified that *M. emiliae*, *Monodelphis* sp. and *Monodelphis* aff. *adusta* seem to have retained the plesiomorphic condition of a simple NOR. Conversely, this condition became variable in *M. domestica* and in the *M. brevicaudata* species complex, which in addition to the NOR identified in the autosomal pair 7, also presents NORs in both chromosomes of the sex pair, indicating a duplication of this site.

In *M. murina*, intraspecific geographic variation in NORs were detected. Specimens from the Purus River have multiple NORs, those collected in the state of Goiás have simple NOR in the short arms of pair 6 (Palma and Yates 1996, Carvalho et al. 2002) and those from the state of Pernambuco present additional markings in the long arms of pairs 3 and 5 (Souza et al. 1990). Furthermore, both specimens from the Purus River differed from the others regarding sex chromosomes.

Our results indicate geographic variation in NORs for *M. demerarae*. Amazonian specimens analysed did not present ribosomal cistrons in the short arms of the fifth pair, as recorded for specimens from the Atlantic forest in the Rio Grande do Sul and São Paulo states of southern Brazil (Carvalho et al 2002, Svartman and Vianna-Morgante 2003, Svartman 2008). Several studies have shown that considerable genetic variation exists among referred populations of this taxon (Voss and Jansa 2003, Dias et al. 2010, Gutiérrez et al. 2010). Therefore, several nominal taxa previously considered synonyms are now treated as valid species. Currently, *M. demerarae* is considered to occur in south to northern and central Brazil, and to southern Bahia (Gardner 2008, Dias et al. 2010) and *Marmosa paraguayana* Tate 1931 occurs from northern border of Espírito Santo state, south to Rio Grande do Sul, and east to Misiones (Argentina), and eastern Paraguay (Gardner 2008). However, some authors consider it to go as far north as Pernambuco state in northeastern Brazil (Voss and Jansa 2003). Thus, considering the geographic dis-

tribution of this taxon, the 18S rDNA data presented for locations in northern and eastern Brazil possibly belong to specimens of *M. paraguayana*. As such, this character would have a cytotaxonomic value, and rearrangements involving the ribosomal sites could be related to speciation events related to this sister-species pair (Gutiérrez et al. 2010).

In *Didelphis marsupialis* from several Amazonian sites, only NOR activity varied, as was already reported in specimens from the Atlantic forest (Yonenaga-Yassuda et al. 1982, Svartman and Vianna-Morgante 2003).

Conclusion

Dutrillaux (1979) suggested that a small sample size would be inadequate for the knowledge of species karyotypes. Heeding this admonition, we used a relatively large number of individuals for each species analysed to uncover a range of variations that most likely would not have been detected had we used fewer individuals per species. The use of integrative analyses and new methodologies, such as taxonomy, phylogeny, and molecular cytogenetics could improve our understanding of the significance of these chromosomic variations. However, for the Amazon region, a significant limitation for cytogenetic studies is still the restricted collection effort, the vast geographical extent of the region and the difficulty of access to remote areas.

The cytogenetic data presented here shows that didelphid marsupial karyotypes present intraspecific variation in the morphology of sex chromosomes and in chromosomic markers (C-band and NOR) and present some geographic variation in the distribution of these features for several species. Furthermore, there are many areas in the Amazon, including the transition zone between the Amazon and the Cerrado biomes, which do not have cytogenetic records for any didelphid species. This situation seriously undermines our understanding of the significance of the recorded variation, whether it is part of a continuous gradient, or whether it represents intraspecific gradations, or whether it is related to new lineages or cryptic species still uncovered. Thereby, despite the chromosomal stability related to diploid numbers and chromosomal formula in marsupials across continents, didelphids present some intra- and interspecific chromosomal variations, probably related to frequent chromosomal rearrangements. Additional systematic sampling and analyses will be required for a better understanding of the karyotypic evolution of this group.

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Supplementary material 1

Voucher specimens

Authors: Carlos Eduardo Faresin e Silva, Rodrigo Amaral de Andrade, Érica Martinha Silva de Souza, Eduardo Schmidt Eler, Maria Nazareth Ferreira da Silva, Eliana Feldberg

Data type: Microsoft Word Document (.docx)

Explanation note: All analyzed specimens were deposited at Mammals Collection in the Instituto Nacional de Pesquisas da Amazônia (INPA); specimens are indicated by species, sampling sites, genus and collector number, followed by INPA collection number (in parentheses) when available.

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RESEARCH ARTICLE

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Dual mechanism of chromatin remodeling in the common shrew sex trivalent (XY_1Y_2)

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Abstract

Here we focus on the XY_1Y_2 condition in male common shrew *Sorex araneus* Linnaeus, 1758, applying electron microscopy and immunocytochemistry for a comprehensive analysis of structure, synapsis and behaviour of the sex trivalent in pachytene spermatocytes. The pachytene sex trivalent consists of three distinct parts: short and long synaptic SC fragments (between the X and Y_1 and between the X and Y_2, respectively) and a long asynaptic region of the X in-between. Chromatin inactivation was revealed in the XY_1 synaptic region, the asynaptic region of the X and a very small asynaptic part of the Y_2. This inactive part of the sex trivalent, that we named the 'head', forms a typical sex body and is located at the periphery of the meiotic nucleus at mid pachytene. The second part or 'tail', a long region of synapsis between the X and Y_2 chromosomes, is directed from the periphery into the nucleus. Based on the distribution patterns of four proteins involved in chromatin inactivation, we propose a model of meiotic silencing in shrew sex chromosomes. Thus, we conclude that pachytene sex chromosomes are structurally and functionally two different chromatin domains with specific nuclear topology: the peripheral inactivated 'true' sex chromosome regions (part of the X and the Y_1) and more centrally located transcriptionally active autosomal segments (part of the X and the Y_2).

Keywords

Sex body, MSCI, synaptonemal complex, γH2AFX, ATR, SUMO-1, ubiH2A, *Sorex araneus*

Introduction

At first meiotic prophase, the male sex chromosomes in mammals form a specific heterochromatic nuclear domain (Solari 1974; Handel 2004). The structure and behaviour of the sex bivalent changes from zygotene to late diplotene. In the majority of mammal species the processes of pairing and synapsis of the X and Y chromosomes at zygotene occurs later than the same processes in autosomes. At early and mid pachytene the sex bivalent is usually located in the centre of the meiotic nucleus. At mid pachytene the sex chromosomes become shorter due to condensation and homologous regions of the X and Y are completely paired (Burgoyne 1982). Recombination nodules appear only in the short pseudoautosomal region (PAR) of the sex bivalent. In many mammals irregular thickenings may occur at asynaptic sites of axial elements of the sex bivalent. After that the sex bivalent gradually moves from the centre of the nucleus to its periphery and forms a so-called XY or sex body (Solari 1974).

The chromatin of the sex chromosomes transforms into an inactive condition and this chromatin remodelling process is known as meiotic sex chromosome inactivation (MSCI) (McKee and Handel 1993; Turner et al. 2000). MSCI is the process whereby unsynapsed regions of the sex chromosomes undergo transcriptional silencing (Lifschytz and Lindsley 1972; Handel and Hunt 1992; Turner et al. 2002, 2007); this is a case of MSUC (meiotic silencing of unsynapsed chromatin) (Schimenti 2005). The asynaptic chromatin undergoes inactivation by incorporation and modification of specific proteins (Burgoyne et al. 2009). First, BRCA1 (breast cancer 1) accumulates in non-synaptic areas of the sex chromosomes, which starts the process of phosphokinase ATR (ataxia telangiectasia- and RAD3-related) recruitment and then there is ATR-dependent phosphorylation of the γH2AFX (phosphorylated (Ser139) histone 2 A.X) histone (Turner et al. 2004). At early pachytene, ubiH2A (ubiquitinated histone H2A), SUMO-1 (small ubiquitin-related modifier-1) and other proteins are incorporated into the asynaptic chromatin of the sex chromosomes (Baarends et al. 2005). Such modification of chromatin decreases its transcriptional activity as confirmed using Cot-1 RNA FISH and RNA polymerase type II immunolocalisation (Turner et al. 2005; Baarends et al. 2005). Thus, the chromatin of the sex body is inactive.

MSCI has been well studied for the normal male sex chromosome system in mammals (XY), but there are few data on this process for multiple sex chromosome systems.

Translocation between the X and an autosome results in the formation of multiple sex chromosomes (XY_1Y_2; where the X is a product of a translocation between the 'true' X and an autosome, Y_1 is the 'true' Y and Y_2 is the autosome). The XY_1Y_2 condition has been demonstrated in insects (Jacobs 2003), fish (Centofante et al. 2006; de Oliveira et al. 2008) and, in particular, among mammals – including marsupials: greater bilby *Macrotis lagotis* (Sharp 1982), and placentals: Indian muntjac *Muntiacus muntjak* (Artiodactyla, Fronicke and Schertan 1997), red brocket deer *Mazama americana* (Artiodactyla, Aquino et al. 2013), big fruit-eating bat *Artibeus lituratus* (Chiroptera, Solari and Pigozzi 1994), short-tailed fruit bat *Carollia perspicillata* (Chiroptera, Noronha et al. 2009), delicate mouse *Salinomys delicates* (Rodentia, Lanzone et al. 2011), Sahel gerbil *Taterillus*

arenarius and Senegal gerbil *Taterillus pygargus* (Rodentia, Ratomponirina et al. 1986; Volobouev and Granjon 1996) and others (see reviews by Fredga 1970; Sharman 1991; and Yoshida and Kitano 2012). An XY_1Y_2 sex chromosome system also characterises species of shrews (small insectivores) belonging to the *Sorex araneus* group (Eulipotyphla; Hausser et al. 1985), including the Eurasian common shrew *Sorex araneus* Linnaeus, 1758 which is a model system for evolutionary cytogenetics with numerous Robertsonian autosomal variants as well as the XY_1Y_2 condition (Searle and Wójcik 1998).

The XY_1Y_2 condition in the common shrew arises from a tandem fusion between an autosome and the true X chromosome (Sharman 1956, 1991; Fredga 1970; Searle et al. 1991) (Fig. 1a). Although the observation of a meiotic sex trivalent was part of the discovery of the XY_1Y_2 condition in the common shrew it was not until the work of Pack et al. (1993) that chromosome pairing in the XY_1Y_2 at meiotic prophase I was first examined. We supplemented those early observations with the discovery that the γH2AFX histone is associated with the true sex chromosome regions of the pachytene sex trivalent (Matveevsky et al. 2012).

In this paper we analyse the distribution of four transcription silencing proteins (ATR, γH2AFX, SUMO-1, ubiH2A) on the sex trivalent XY_1Y_2 at prophase I in common shrew spermatocytes and assess how these participate in MSCI.

Material and methods

Shrews. A total of five adult males of the common shrew were collected from a locality in the vicinity of the Moscow-Neroosa chromosomal hybrid zone (near Ozyory town, Moscow Region) in April 2014, at the beginning of the breeding season. All animals were karyotyped using the method of Pavlova et al. (2008), with modifications. The trypsin-Giemsa staining technique of Král and Radjabli (1974) was used for identification of chromosome arms by G-bands, following the standard nomenclature for the *S. araneus* karyotype, which uses letters of the alphabet for chromosome arms (Searle et al. 1991).

All karyotypes were characterised by the set of invariant autosomal metacentrics *af*, *bc*, *jl* and *tu* as well as the XY_1Y_2 sex chromosomes system. Race-specific autosomes differed between individuals, two males had *gm*, *hi*, *kr*, *no* and *pq* metacentrics which mark the karyotype of the Moscow race. Other males had *go, hi, kr, mn* and *pq* metacentrics which characterise the Neroosa race. All shrews had the same diploid number of chromosomes (2n=21). Spermatocyte spreads were obtained from all males. All necessary national and institutional guidelines for the care and use of animals were followed.

A total of 331 cells were analysed of which 14 were prepared for electron microscopy and 317 for fluorescence microscopy. All the latter were labelled with SYCP3 (synaptonemal complex protein 3) and CREST and a proportion of cells were labelled with other antibodies (γH2AFX: 90; SUMO-1: 59; ubiH2A: 52; ATR: 32; MLH1: 74; SYCP1: 28; RNA Pol II: 10).

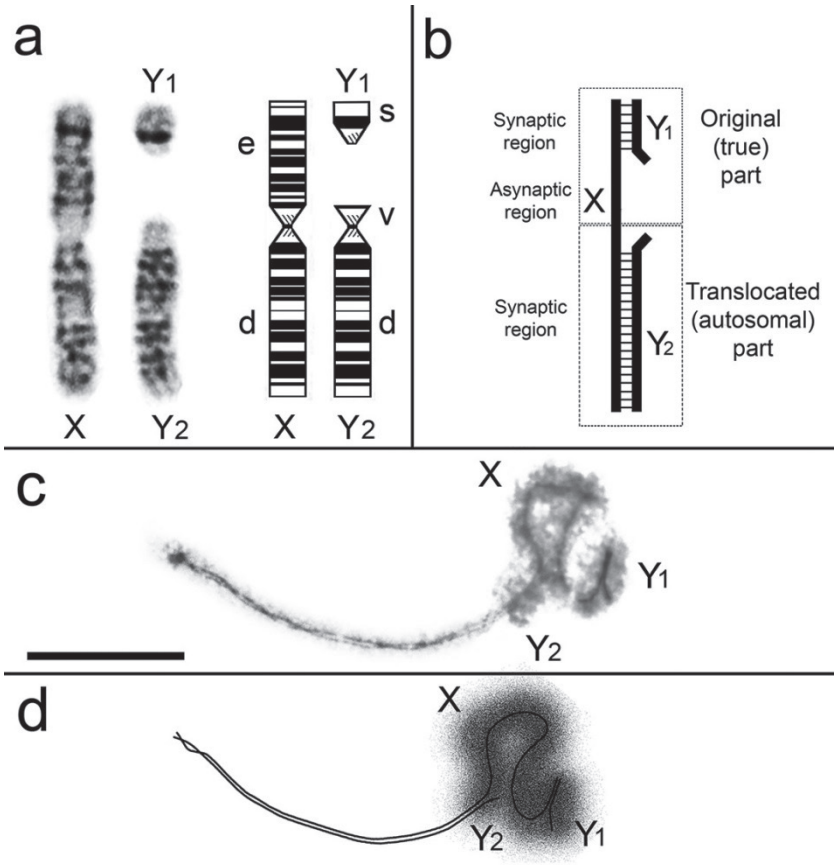

Figure 1. a G-banded sex chromosomes in the male common shrew (left) and ideogram with chromosome arms labelled according to the alphabetic nomenclature of Searle et al. (1991) **b** Schematic diagram of the shrew pachytene sex trivalent, based on Pack et al. (1993) and our data **c** Electron micrograph of a shrew sex trivalent, XY_1Y_2 at late pachytene. The true X region and the Y_1 are surrounded by electron-dense material. Scale bar: 5μm. **d** Diagram of the XY_1Y_2 configuration as represented in Fig. 1c.

Meiotic spread preparations. Synaptonemal complex (SC) preparations were made and fixed using a previously described technique (Kolomiets et al. 2010). Ag-NO_3-stained slides were screened under a light microscope to select suitably spread cells. Once selected, plastic (Falcon film) circles were cut out with a diamond tip and transferred onto grids and examined in a JEM 100B electron microscope.

Antibodies, immuncytochemistry and multistep immunostaining procedure. Poly-L-lysine-coated slides were used for immunostaining. The slides were placed in phosphate buffer saline (PBS) and incubated overnight at 4°C with the primary antibodies diluted in antibody dilution buffer (3% bovine serum albumin - BSA, 0.05% Triton X-100 in PBS): mouse anti-MLH1 (1:50–1:100, Abcam, Cambridge, UK), rabbit polyclonal anti-SYCP1 (1:500, Abcam, Cambridge, UK), rabbit polyclonal anti-SYCP3

(1:500–1:1000, Abcam, Cambridge, UK), mouse monoclonal anti-ATR (1:200, Abcam, Cambridge, UK), human anticentromere antibody CREST (Calcinosis Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly, and Telangiectasia) (1:500, Fitzgerald Industries International, Acton, MA, USA), mouse monoclonal anti-SUMO-1 (1:250, Zymed Laboratories, South San Francisco, CA, USA), mouse monoclonal anti-ubiquityl histone H2A (1:400, Millipore, Billerica, MA, USA), and mouse anti-phospho-histone H2AX (also known as γH2AFX) (1:1000, Abcam, Cambridge, UK).

After washing, we used the following corresponding secondary antibodies diluted in PBS: FITC-conjugated bovine anti-rabbit IgG (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-rabbit Alexa Fluor 488 (1:500, Invitrogen Corporation, Carlsbad, CA, USA), FITC-conjugated horse anti-mouse IgG (1:500, Vector Laboratories, Burlingame, CA, USA), Rodamin-conjugated chicken anti-rabbit IgG (1:400, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-human Alexa Fluor 546 (1:500, Invitrogen Corporation, Carlsbad, CA, USA), goat anti-mouse Alexa Fluor 546 (1:200, 1:1000, Invitrogen Corporation, Carlsbad, CA, USA).

Immunostaining was carried out sequentially in 3 steps: 1. SYCP3/CREST (or SYCP1/MLH1); 2. ATR (or SUMO-1 or ubiH2A); 3. γH2AFX. After an each step slides were washed in PBS (6–7 times for 7–10 min) and mounted with Vectashield mounting medium containing 4,6-diamino-2-phenylIndol (DAPI) (Vector Laboratories, Burlingame, CA, USA). Slides were examined using an Axioimager D1 microscope (Carl Zeiss, Jena, Germany) equipped with an Axiocam HRm CCD camera. Images were processed using Adobe Photoshop CS3 Extended.

It should be noted that after photobleaching, bound antibodies of the first round still remain attached to the cellular structures. The more antibodies attached to the structures of interest the higher the probability that epitopes of further rounds of immunolocalisation become inaccessible. To ensure that these processes have not impacted our results, we performed control experiments for all antibodies.

Controls. We always conducted parallel control experiments on different slides when immunostaining was performed with a single antibody to a MSCI specific protein (double immunostaining). Our colleague Dr TM Grishaeva has conducted a bioinformatics analysis of the proteins studied. The pairwise sequence alignment of human and mouse proteins, which was performed by the COBALT program (NCBI), demonstrated high conservation of the H2AX, ubiH2A, SUMO-1, ATR and Polo II proteins. Comparison of the proteins did not reveal any problematic similarity between them. The pairwise sequence alignment of ATR and H2AX showed no amino acid sequence similarity. SUMO-1 and H2AX appeared to have 14 coincidences of amino acids, which should not affect the cross-reaction. ubiH2A and H2AX have a high level of similarity except a short sequence in the carboxyl terminus. Nevertheless, an analysis of the fluorescence intensity profile suggests a close, but not identical, picture of distribution for ubiH2A and H2AX (Matveevsky et al. 2016).

Image analysis. Intensity Correlation Analysis (ICA) was carried out according to Reitan et al. (2012). Scatter plots, Pearson's coefficients (p_r) and overlap correlation coefficients (r) were obtained using a plug-in ICA (Li et al. 2004) of ImageJ 1.45 (Ras-

band 1997–2016). p_r helps to evaluate the degree of correlation between the different intensities and is ranked from -1 (negative correlation) to +1 (positive correlation) (French et al. 2008). In analysing scatter plots, overlaying green and red signal resulted in a yellow signal. The more yellow in the scatter plot, the higher the level of overlap. The width of the yellow signal distribution in scatter plots corresponded to the degree of co-localisation of the fluorescence signals being compared: the wider the distribution of the signal, the higher the level of overlap of the two channels.

To evaluate the degree of co-localisation of some proteins, we have developed Fluorescent-Intensity Profiles (FIPs) using the ImageJ plug-in RGB profiler (created by Christophe Laummonerie, Jerome Mutterer, Institute de Biologie Moleculaire des Plantes, Strasbourg, France) and following Barak et al. (2010) and Fargue et al. (2013).

Statistical analysis. All of the data are shown as the mean values ± SD. Student's *t*-test was performed to determine significant differences in the data. All statistical analyses were conducted using GraphPad Prism Version 5.0 (GraphPad Software, CA, USA).

Results

Synapsis and markers of recombination of the XY_1Y_2 configuration at pachytene

The sex trivalent XY_1Y_2 was detected in spermatocyte nuclei from the beginning of the early pachytene stage in electron micrographs. Three distinct parts are clearly visible on the sex trivalent: short and long synaptic SC segments and a long asynaptic segment of the X chromosome arranged between them. The first (short) segment of the SC (the PAR synaptic site) is formed between the true X region and the Y_1 and is always located at the periphery of a nucleus. The second (long) segment is the SC between the translocated (autosomal) part of the X chromosome and the Y_2 (Fig. 1); this fragment is always directed into the spermatocyte nucleus. The axial element of the X chromosome is irregularly thickened in the asynaptic region that sits between the two synaptic regions.

At the early stages of prophase I, the length of the SC between the autosomal part of the trivalent (X and Y_2) is variable. At late zygotene and early pachytene, synapsis was observed along the entire length of the segment; while in mid pachytene desynapsis of chromosome arm *v* of Y_2 (Fig. 1a) was detected. The length of this desynaptic segment was about 3-4% of the total length of Y_2 (Fig. 1b).

At mid-late pachytene, a cloud of electron-dense material overlays the true sex chromosome regions which include the region of XY_1 synapsis, the asynaptic part of the X chromosome, a short pericentromeric segment of the SC between the X and Y_2 and the asynaptic part of the Y_2 (Fig. 1c–d). Thus, it is precisely this part of the XY_1Y_2 that takes the form of the typical sex body in male mammals.

Immunostaining with antibodies against the proteins of the axial (SYCP3) and central (SYCP1) elements of the SC revealed the differences in the distribution pat-

terns of these proteins in the sex trivalent structure. SYCP3 and SYCP1 foci were always displayed evenly and clearly on the long synaptic SC (between the Y_2 and translocated part of the X), while the distribution foci of these proteins were either fragmentary (Fig. 2a, f, k) or completely absent in the case of SYCP1 (Fig. 2p, p', p") on the short PAR synaptic fragment of SC (between the Y_1 and the true X region).

Centromeres of the sex trivalent were detected using CREST serum. One centromere was located on the Y_1 acrocentric and a second was seen where the X and the Y_2 associated. Sometimes two centromeric signals were detected in this long synaptic fragment of the SC. Thus, localisation of the X and Y_2 centromeres in the structure of the sex trivalent does not coincide.

Late recombination nodules were detected using antibodies to MLH1 (MutL homolog 1; a DNA mismatch repair protein component that is specific to these nodules). In the structure of the sex trivalent one MLH1 focus is located on the short PAR synaptic site (where the Y_1 and the true part of X pair) and another one where the Y_2 and translocated part of X pair (Fig. 2q).

MSCI markers distribution in the pachytene XY_1Y_2

The distribution of the four transcriptional silencing proteins was analysed using immunostaining. ATR had a discontinuous localisation in the true sex chromosome regions, including a few ATR foci in the region of XY_1 synapsis (Fig. 2a–e).

As a rule, as shown in our previous work on common shrews (Matveevsky et al. 2012), γH2AFX is also associated with the true sex chromosome regions within the XY_1Y_2, including chromatin of the asynaptic region of the X chromosome. It should be noted that the histone γH2AFX extends into the autosomal centromeric region of the XY_1Y_2 (Figs 2c, h, m, 3).

SUMO-1 is also localised only in the true sex chromosome regions, adjacent to the axial elements of the sex trivalent. Unlike the continuous distribution of γH2AFX, SUMO-1 has a granular pattern of localisation. The chromatin of the translocated part of XY_1Y_2 does not become immunostained with antibodies to the SUMO-1 (Figs 2f–j, 3).

Localisation of ubiH2A looks like an extensive cloud around the true X chromosome and Y_1 only without extending to the autosomal part of the XY_1Y_2 (Figs 2k–o, 3).

ICA and FIPs allowed us to estimate the degree of MSCI protein co-localisation (Fig. 2c', h', m'). This was high for γH2AFX and ubiH2A (r_p = 0.86±0.06, r = 0.92±0.04; n=22) (see Fig. 4). Regarding the FIPs, the γH2AFX-signal path was similar to the ubiH2A-signal path, but slightly wider in coverage (Fig. 2m'). The degree of γH2AFX / SUMO-1 co-localisation was lower (r_p = 0.76±0.09, r = 0.86±0.07; n=30) (see Fig. 4). The SUMO-1 signal occupies a narrower part of the X axis and shows three peaks within the chromatin around the XY_1 pairing region (Fig. 2h'). A significant low degree of co-localisation was found for the γH2AFX / ATR pair (r_p = 0.38±0.08, r = 0.58±0.09; n=10), as evident in Fig. 2c (see Fig. 4). The ATR-signal path has two peaks

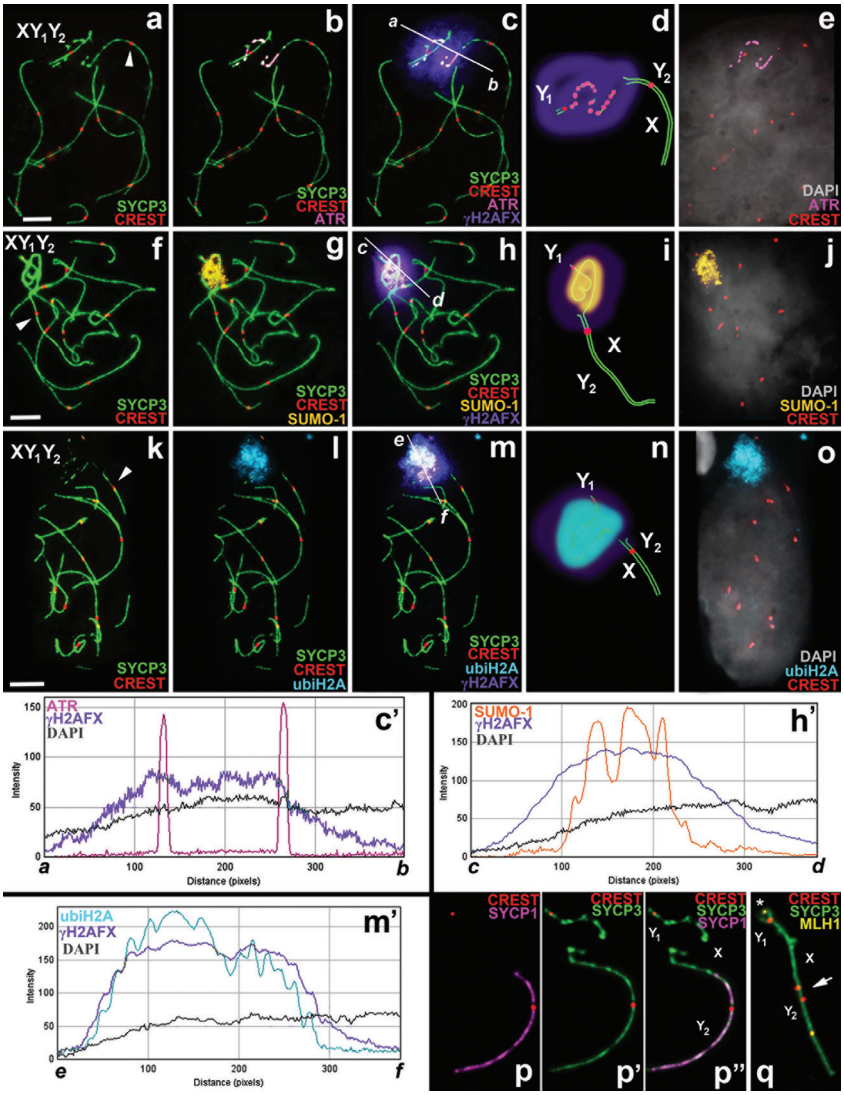

Figure 2. Mid-pachytene spermatocytes and male sex (XY_1Y_2) chromosomes of *Sorex araneus*. Bar = 5μm. The axial elements of the SC and the kinetochores were localised using anti-SYCP3 (*green*) and anti-CREST (*red*) antibodies, respectively. **a–e** ATR (*magenta*) has a discontinuous localisation within the chromatin of the true sex chromosome regions (part of the X and the Y_1). The co-localisation of ATR, γH2AFX (*violet*), DAPI (*grey*) is shown in graph *a-b* (see **c** and **c'**) **f–j** SUMO-1 (*yellow*) is localised on the chromatin of true sex chromosome regions. The co-localisation of SUMO-1, γH2AFX (*violet*) and DAPI (*grey*) is shown in graph *c-d* (see **h** and **h'**) **k–o** ubiH2A (*cyan*) is localised on the chromatin of the true sex chromosome regions. The co-localisation of ubiH2A, γH2AFX (*violet*) and DAPI (*grey*) is shown in graph *e-f* (see **m** and **m'**) **d, i, n** Diagrams of the sex trivalents **p, p', p''** SYCP1 (*magenta*) is located on the area of chromosome synapsis of the autosomal part of the XY_1Y_2 (from **a-c**) **q** XY_1Y_2 has two MLH1 signals (*yellow*). The MLH1 signal within the PAR synaptic site is marked by an asterisk. The arrowhead indicates the centromeres of the autosomal part of sex trivalent (part of the X and the Y_2) which are not co-oriented with each other (*red*).

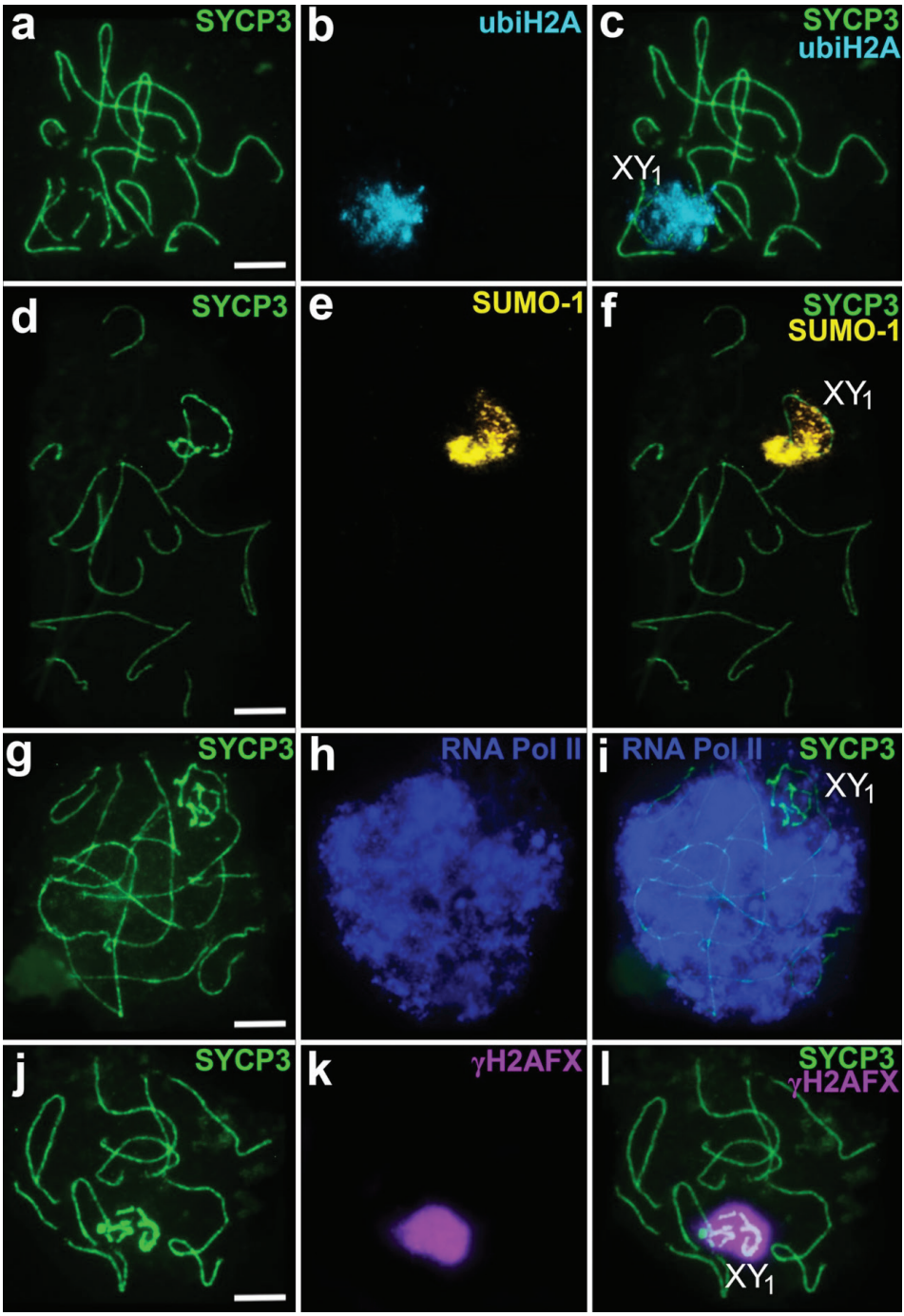

Figure 3. Mid-pachytene spermatocytes of *Sorex araneus*. Double immunostaining with antibodies: **a–c** anti-SYCP3 (*green*)/anti-ubiH2A (*cyan*) **d–f** anti-SYCP3 (*green*)/anti-SUMO-1 (*yellow*) **g–i** anti-SYCP3 (*green*)/anti-RNA Pol II (*blue*) **j–l** anti-SYCP3 (*green*)/anti-γH2AFX (*violet*). The true sex chromosome region is designated as XY_1. Scale bars: 5 μm.

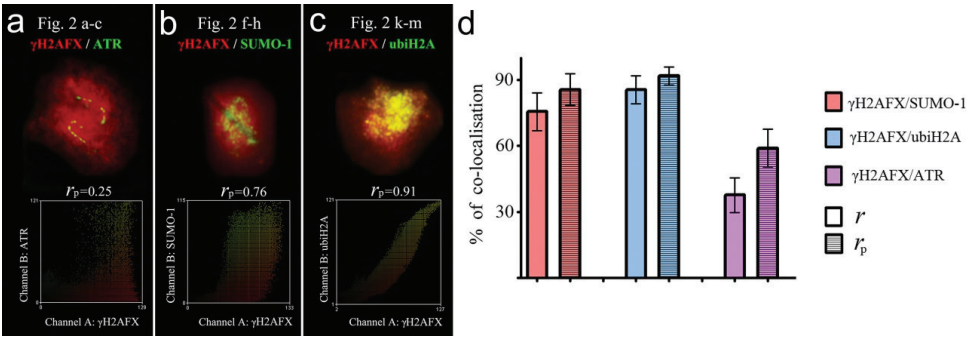

Figure 4. Intensity correlation analysis (ICA) represented by scatter plots showing the paired intensities of two channels (**a** γH2AFX - ATR, Fig. 2a–c **b** γH2AFX - SUMO-1, Fig. 2f–h **c** γH2AFX - ubiH2A Fig. 2k-m). r_p - Pearson correlation coefficient. See more details in the text. Degree of co-localisation for signals in sex trivalents of common shrew (**d**). On the *y*-axis, the percentage of co-localised signals are shown according to overlap correlation coefficients (*r*) and the Pearson correlation coefficient (r_p).

in the sites of the crossing ATR- and SYCP3-signals and is not synchronised with the γH2AFX-signal path (Fig. 2m').

The RNA Pol II intensively immunostained the whole nucleus, except for the zone where the true part of the sex trivalent is located. In this area the signal is reduced (Fig. 3g–i).

Discussion

Specific features of synaptic and recombination behaviour of the XY_1Y_2 at pachytene

The sex chromosomes (XY_1Y_2) in the common shrew were originally described by Sharman (1956). Later studies of total preparations of SC by light microscopy did not reveal details of XY_1Y_2 synapsis at prophase I (Wallace and Searle 1990; Mercer et al. 1992); these were described using electron microscopy (Pack et al. 1993; Narain and Fredga 1997). It was found that the sex trivalent forms an argyrophilic sex body that moves to the nucleus periphery during prophase I. It is interesting that the autosomal part of the sex trivalent is directed into the meiotic nucleus. A similar synapsis of sex chromosomes and the formation of electron-dense material around the true sex chromosome regions within the XY_1Y_2 trivalent were identified previously in the bat *Artibeus lituratus* (Solari and Pigozzi 1994) and the deer *Mazama americana* (Aquino et al. 2013). A similar pattern of synapsis in the sex trivalent was also detected in some species of gerbils (Wahrman et al. 1983; Ratomponirina et al. 1986) and in the muntjac; however, in the last case it was difficult to identify clearly the synaptic participants in the absence of electron micrographs (Pathak and Lin 1981).

Desynapsis of the short peritelomeric segment of Y_2 within the sex trivalent (i.e. chromosome arm *v*: Fig. 1a) has previously been described for several species including the aforementioned bats, deer and gerbils and Pack et al. (1993) already mentioned

this phenomenon for the common shrew. From G-banding it looks as if the chromosome arm *v* on the Y_2 is homologous to an equivalent region on the X chromosome (Fig. 1a). Thus, the desynapsis may be an unusual behaviour of homologous chromatin in proximity to the chromosomal breakpoint of the X-autosome tandem fusion. However, further studies are needed to establish whether the chromosome arm *v* on the Y_2 is truly homologous to the equivalent region on the X chromosome.

Our data show that each part of the XY_1Y_2, the true sex chromosome regions and the translocated parts, displayed one signal of a recombination nodule. A similar pattern of recombination events was revealed previously in common shrew spermatocytes (Borodin et al. 2008) but sometimes these authors visualised two MLH1 signals on the autosomal part of the trivalent, although there usually was a single signal. So, in general features, our results confirmed previous data.

Chromatin remodelling in the pachytene XY_1Y_2

The study of chromatin remodelling of the sex body is possible by immunodetection of specific epigenetic MSCI markers, such as BRCA1, ATR, γH2AFX, SUMO-1 and ubiH2A (Mahadevaiah et al. 2008; Manterola et al. 2009; Page et al. 2012; Sciurano et al. 2012, 2013; Matveevsky et al. 2016; and others). It has previously been found that ATR, γH2AFX, SUMO-1 and ubiH2A play some role in maintaining an inactive form of the chromatin and, in general, in the formation of the sex body (Moens et al. 1999; Mahadevaiah et al. 2001; Rogers et al. 2004; Cao and Yan 2012). In shrew spermatocytes, MSCI starts with the appearance of ATR in the asynaptic region of the X chromosome. After that, the second wave of γH2AFX phosphorylation covers the chromatin associated with the true sex chromosome regions, as shown in our previous work (Matveevsky et al. 2012). Both SUMO-1 and ubiH2A appear simultaneously on the sex trivalent. This picture of MSCI is typical for the XY chromosomes of most mammals, including rodents (Turner 2007; Namekawa and Lee 2009). But the chromatin of the shrew sex trivalent has some distinguishing features, for example, ATR and SUMO-1 are narrowly localised along the axial/lateral elements in both the XY_1 synaptic region and the asynaptic region within the sex trivalent. We have not seen the spread of the ATR signal into the surrounding chromatin. In contrast, in mice ATR is immunostained along the asynaptic elements with a less intense signal extending into the surrounding chromatin (Turner et al. 2004; Manterola et al. 2009; Fedoriw et al. 2015) and in the mole vole an intense ATR signal surrounds the entire sex bivalent (Matveevsky et al. 2016). SUMO-1 covers the asynaptic region as an extensive cloud in mice (La Salle et al. 2008; Manterola et al. 2009). At the same time, γH2AFX and ubiH2A are as widely distributed over the shrew sex chromatin as in mice and other species (de la Fuente et al. 2007; Sciurano et al. 2012, 2013). Although the chromatin organisation in mammals is universal, a special feature of the epigenetic landscape of sex chromatin has been shown in horses (Baumann et al. 2011) and in human (Metzler-Guillemain et al. 2008). In this case γH2AFX does not cover the chromatin

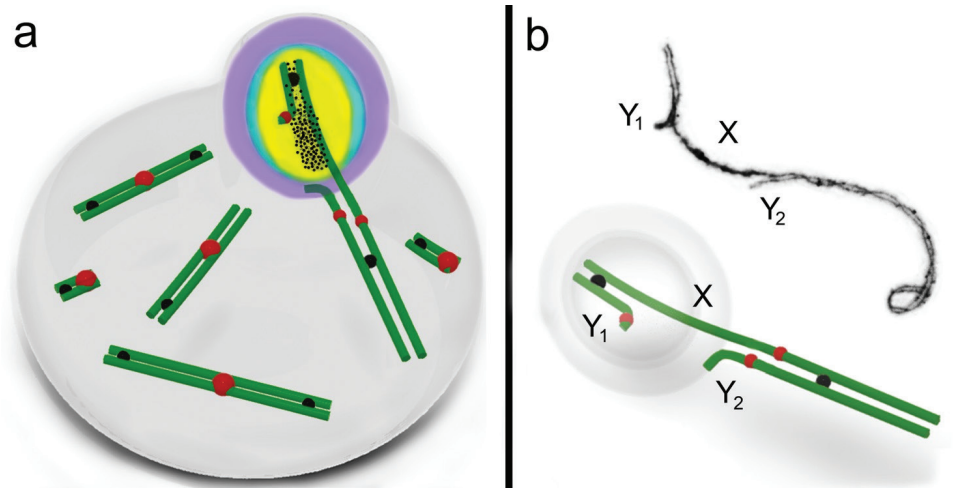

Figure 5. Schematic illustration of male common shrew MSCI. A mid-pachytene spermatocyte (**a**) and a sex (XY_1Y_2) trivalent (**b**) of a shrew are shown. An electron micrograph of the sex trivalent is shown at the top of the **b**. The true sex chromosome regions (part of the X and the Y_1) form a sex body on the periphery of the nucleus. The chromatin of the sex body undergoes reorganisation. MSCI markers have different distributions: SUMO-1 (*yellow*), ATR (*black dots*), ubiH2A (*blue*), γH2AFX (*violet*). ATR is localised on the true sex chromosome regions, and is especially intense on the asynaptic region with a smaller amount where there is synapsis. SUMO-1 and ubiH2A are localised on both the asynaptic and synaptic regions of the true sex chromosome regions. γH2AFX overlays all the true sex chromosome regions and the unpaired part of the Y_2 axial element. Representative autosomal SCs are shown. MLH1 signals are shown as black balls. The red balls indicate centromeres.

but is localised to the axial elements of the sex bivalent, while ubiH2A is completely absent from the sex body. It is obvious that different epigenetic markers of MSCI may be species-specific features. It is worth noting that we analysed the distribution of the mouse monoclonal ubiH2A, E6C5 clone, while the rabbit monoclonal ubiH2A, D27C4 clone, generates different results (Hasegawa et al. 2015).

The proteins around the true sex chromosome regions of the XY_1Y_2 are argentophilic and so the electron-dense cloud is detected around the site of synapsis between X and Y_1, the unpaired region of the X chromosome, the desynaptic part of the Y_2 and a short pericentromeric synaptic site between X and Y_2 (Fig. 1a–d).

On the basis of immunocytochemistry of MSCI proteins, in this study we suggest a chromatin remodelling model in shrew pachytene spermatocytes (Fig. 5), including two different structural and functional chromatin domains within the sex trivalent: the inactivated chromatin of the true sex chromosome regions and the absence of inactivation in the translocated part. The true sex chromosome regions within the sex trivalent form a macrochromatin domain with both universal and specific features of MSCI, while the translocated part is a typical autosomal chromatin domain. Our interpretations are strongly supported by the distributions of proteins as observed in the preparations, with substantial replication and care in immunostaining and no indications

of artefacts that are always a possibility with the spreading technique and efficiency of antibody affinity/sensitivity.

It is worth noting that Pack et al. (1993) assumed, without firm evidence, that the translocated component of the XY_1Y_2 in common shrews does not likely undergo inactivation; similar assumptions have been made for other species such as the sex trivalent in the big fruit-eating bat (Solari and Pigozzi 1994). We have been able to use immunological markers to demonstrate that the autosomal component of the sex trivalent (excluding the unpaired part of Y_2) in the common shrew remains free of the chromatin modifications associated with MSCI.

Thus, our study shows that the shrew sex trivalent (XY_1Y_2) has a similar scenario of synapsis and meiotic silencing of unsynapsed chromatin (MSCI) processes as found in the usual sex chromosomes (XY) of male mammals. Apparently, this particular X-autosome translocation does not change the behaviour of the true sex chromosome regions in meiosis and does not affect the process of chromatin transformation at prophase I.

Thus, we may conclude that remodelling of sex chromatin in shrew spermatocytes neatly fits into the MSCI concept.

Conclusion

A pronounced difference in the structure, behaviour and MSCI of the two parts of the shrew sex trivalent has been revealed on the basis of detailed analysis of the organisation and behaviour of XY_1Y_2 at prophase I of meiosis. The 'head' part of the trivalent that moves to the periphery of the pachytene nuclei involves the true sex chromosome regions and includes synapsis between the X and Y_1 chromosomes. The 'tail' part involves the region of synapsis between the translocated X and Y_2 chromosomes. The structure and behaviour of the 'head' part (true X region and the Y_1) including specific MSCI shows patterns which are typical for a male sex bivalent of mammals. At the same time, the 'tail' part (the translocated region of the X and the Y_2) is located among other autosomes and does not differ from them morphologically excluding the fact that this part is attached to the 'head' part of the sex trivalent. These dual properties of the 'head' and 'tail' parts of the XY_1Y_2 trivalent in shrew spermatocytes are a notable feature of this system.

It is also noteworthy in this study that we have determined for the first time specific features of MSCI related to the discontinuous distribution of ATR along the SC at the site of synapsis between X and Y_1 and the distribution limits of SUMO-1 which occurs in the same part of the SC.

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RESEARCH ARTICLE

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DRAWID: user-friendly java software for chromosome measurements and idiogram drawing

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Abstract

An idiogram construction following chromosome measurements is a versatile tool for cytological, cytogenetic and phylogenetic studies. The information on chromosome length, centromere index and position of cytogenetic landmarks along with modern techniques (e.g. genomic and fluorescence in situ hybridization, banding, chromosome painting) can help to shed light on genome constitution, chromosome rearrangements and evolution. While idiogram construction is a routine task there are only few freely available programs that can perform chromosome measurements and no software for simultaneous measuring of chromosome parameters, chromosomal landmark and FISH signal positions and idiogram construction. To fill this gap, we developed DRAWID (DRAWing IDiogram), java-based cross-platforming program for chromosome analysis and idiogram construction. DRAWID has number of advantages including a user-friendly interactive interface, possibility for simultaneous chromosome and FISH/GISH/banding signal measurement and idiogram drawing as well as number of useful functions facilitating the procedure of chromosome analysis. The output of the program is Microsoft XL table and publish-ready idiogram picture. DRAWID and the manual for its use are freely available on the website at: http://www.drawid.xyz

Keywords

Karyotyping, idiogram, chromosome software, plant cytogenetics

Introduction

Chromosome number, morphology and organization are important parameters for comparative cytogenetic and phylogenetic studies (Mandáková and Lysak 2008; Peruzzi et al. 2009; Cheng et al. 2013; Kirov et al. 2014; Divashuk et al. 2014; Bolsheva et al. 2015; Astuti et al. 2017). Differences in chromosome morphology between individual species are the result of inter- and intra-chromosomal rearrangements which are major forces of evolution and speciation (Rieseberg 2001; De Storme and Mason 2014; Mandáková et al. 2015). Knowledge about chromosome rearrangements and basic chromosome characteristics (e.g. centromere index, arm ratio, relative length, chromosomal asymmetry) can also be useful for the integration of physical and genetic maps, the study of speciation and evolution and for tracing desirable traits during plant breeding processes (Peruzzi and Eroğlu 2013; Budylin et al. 2014; Laskowska et al. 2015; Astuti et al. 2017). Modern molecular cytogenetic techniques, e.g. genomic in situ hybridization (GISH), further help to shed light on karyotype constitution and chromosomal rearrangements (Van Laere et al. 2010; Laskowska et al. 2015). Chromosome number and structure are typically schematically represented in an idiogram, showing chromosome length, centromere index and chromosome arm ratio. Measurements on at least 5 metaphase plates are used to build an idiogram. Moreover, to determine the correct chromosome order and homologous pairs, additional chromosomal markers, such as banding patterns and FISH signals, are often required. For plant species like *Arabidopsis thaliana* (Linnaeus, 1753) Heynhold, 1842 , wheat and maize (*Triticum aestivum* Linnaeus, 1753 and *Zea mays* Linnaeus, 1753, respectively), a well-defined karyotype and idiogram have been published, facilitating cytogenetic studies of their genomes (Gill et al. 1991; Fransz et al. 1998; Sadder and Weber 2001; Badaeva et al. 2007). However, for most plant genera this information is lacking and chromosome measurements are needed to build a karyotype. In addition, cytology-based ecological studies of genome variability require measurements of chromosomes from a large number of individuals.

To accelerate karyotype studies in plants only few software programs are available, including MicroMeasure (Reeves and Tear 2000), IdeoKar (Mirzaghaderi and Marzangi 2015) and KaryoType (Altınordu et al. 2016). These programs allow measurement of chromosome parameters such as centromere index, arm length and ratio, asymmetry index, etc. However, none of these programs is able to simultaneously measure chromosome parameters and chromosomal landmark positions (e.g. band, FISH and GISH signals), allowing idiogram construction.

Here, we present the DRAWID (**DRAW**ing **ID**iogram) – program for chromosome analysis and idiogram construction. DRAWID is a user-friendly and freely available (under GNU General Public License) java-based software program that facilitates basic as well as ISH-based karyotype analysis. DRAWID is equipped with an intuitive graphical user interface. Input files for DRAWID are image files (JPEG and PNG) or

data tables generated by DRAWID itself. Output of the program are Microsoft XL (2010) tables, containing measurement details (centromere index, arm ratio, relative and absolute length of chromosome and chromosome arms, signal and band positions and size (if available), and DRAWID-built idiogram pictures. The idiogram parameters can be easily adjusted to prepare a high-quality image suitable for publication. In addition, to facilitate high-throughput karyotyping the program enables to collect data from different metaphases, and construct an average idiogram with error bars representing the standard deviation for chromosome length and centromere position.

We designed a web page on the website of the Russian State Agrarian University-MTAA (Department of plant genetics, biotechnology and breeding) from which DRAWID v0.26 can be downloaded, together with the manual for its use and possibility for bug reports (http://www.drawid.xyz).

Material and methods

Software development

The original code of the program was written in Java 8 using IntelliJ IDEA as the integrated development environment and is compatible with any Java-enable system with a runtime level of ≥1.7. Microsoft Excel version 10.0 or higher is required.

Abbreviations

FISH Fluorescent in situ hybridization
PCR Polymerase chain reaction
GISH Genomic in situ hybridization

FISH and GISH

For cytogenetic experiments chromosomes were prepared using the SteamDrop method (Kirov et al. 2014). To visualise 5S and 45S rDNA in *Allium fistulosum* Linnaeus 1753, the plasmids pSct7 (Lawrence and Appels 1986) and pTa71 (Gerlach and Bedbrook 1979) were used. FISH was performed as described in Kirov et al. (2016). Biotin and digoxigenin labeled probes were detected by Streptavidin-Cy3 (Sigma-Aldrich, USA) and anti-digoxigenin-FITC (Roche, Germany), respectively.

GISH on × *Festulolium* Ascherson & Graebner, 1902 hybrids was performed as described in Van Laere et al (2010). *Lolium perenne* Linnaeus, 1753 was used as probe DNA, labelled with Digoxigenin, while *Festuca pratensis* Hudson, 1762 was used as block DNA.

Microscopy and image analysis

Images were taken by a Zeiss AxioImager M1 fluorescence microscope (400× and 1000× magnification) equipped with an AxioCam MRm camera and ZEN software (Zeiss, Zaventem, Belgium).

Results and discussion

Implementation

DRAWID contains two main modules: (1) idiogram manipulations and (2) chromosome measurements (Figure 1A). DRAWID can draw an idiogram using data from the second module as well as from DRAWID generated Microsoft XL output tables. The first module provides an interface for idiogram manipulation, storage, analysis as well as for data representation. Chromosome order, name, and color and centromere color can be changed using the top menu and the pop-up menu after chromosome selection. Once idiogram adjustments are performed, the idiogram can be added to the DRAWID storage to use it later again. From this window high quality pictures can be saved in PNG format. Data from DRAWID can be saved as XL Microsoft files (.xlsx). The excel data files contain two sheets in total, one sheet with chromosome parameters such as chromosome length, centromere index (short arm length × 100 / total chromosome length), relative chromosome length, short arm and long arm lengths, and one metadata sheet containing information about signal and band positions and size. Building an idiogram for cytogenetically uncharacterized species requires an average idiogram based on measurements of several metaphases. To facilitate this process we implemented a function to build an average karyotype from multiple datasets stored in the DRAWID storage. After application of this function measurements of chromosomes with the same names will be used to calculate means for centromere index, whole chromosome length, short and long arm lengths and standard deviation. An average idiogram is plotted with error bars representing the standard deviation. This idiogram can again be exported and saved either as an image file or as a table. To build idiograms for haploids we inserted a function to merge neighboring (chromosome ordering is just performed by length) chromosomes depending on the ploidy level.

The second module (Figure 1A, B) allows chromosome measurements as well as chromosomal landmarking and includes a number of useful tools to simplify the measurement process. Chromosome and landmark names appear on the picture once the measurement is completed. FISH signal positions can be drawn on the idiogram. Correct positioning of the signals on the idiogram is becoming annoying when many probes and/or loci are involved. Therefore, DRAWID is equipped by a set of functions to correctly position all the signals on the idiogram and to easlily change their colors and names using the icons in the top panel menu. Chromosome name changes in the idiogram are automatically and immediately synchronized with the measurements on the picture. In addition, the visual-

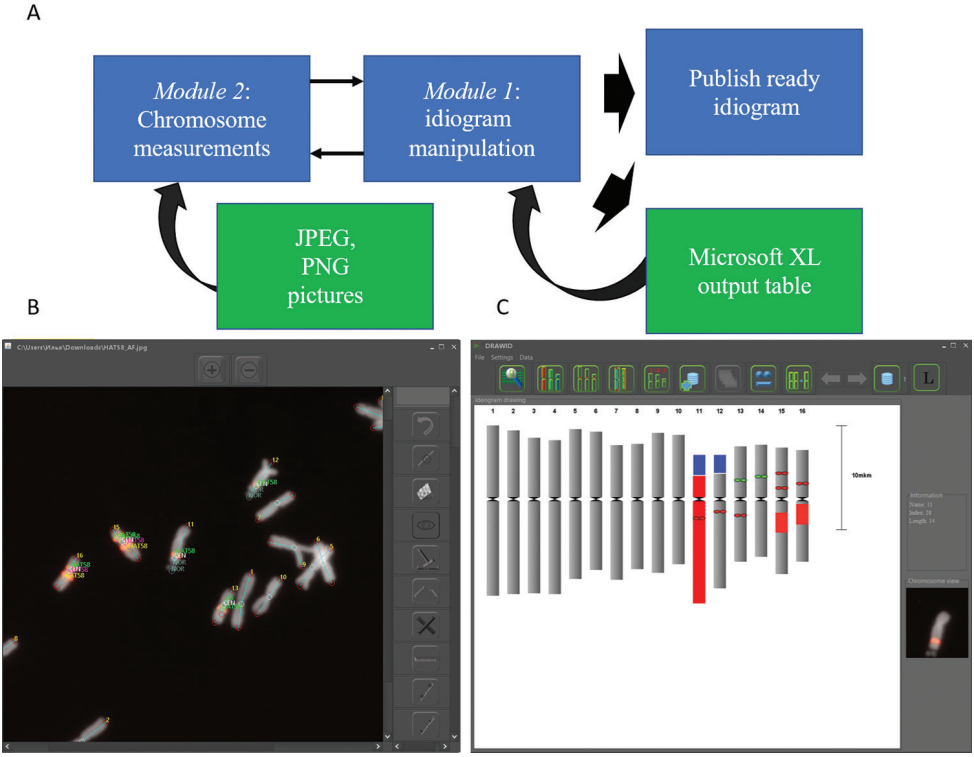

Figure 1. Structure (**A**) and main windows of DRAWID (**B, C**). **B** Interface of chromosome measurement window, containing useful tools for chromosome and FISH/GISH signal measurements. The photograph in this panel shows the result of a FISH experiment on chromosomes of *Allium fistulosum* with biotin-labeled 5S rDNA and HAT58 repeat (Kirov et al. 2017). Lines and signatures show the path of chromosome and signal measuring **C** Interface for idiogram manipulation. This panel shows the idiogram of *A. fistulosum* constructed based on chromosome measurements and FISH (5S rDNA and HAT58 tandem repeat) signal positions in panel **B** Buttons at the top of the panel are used for chromosome and centromere color changing, display legend, chromosome order correction and idiogram storage manipulation. When a chromosome in the constructed idiogram is selected (entire chromosome 11 highlighted in red the image of the selected chromosome along with the parameters of its measurement appear on the screen (on the right of the panel **C**).

ized chromosome on the right side of the panel helps to identify the chromosomes and to link the idogram to the original picture. Sometimes, chromosomes have fragile sites and as a consequence, different fragments of these chromosomes can be positioned on different locations in the metaphase. To virtually join two or more fragments of the same chromosome and build an idiogram, we also implemented a specific function.

All measurements can be scaled by measuring the scale bar in the picture and using the scale bar button. As some errors can occur during measurements, DRAWID has several functions to remove measurements of certain chromosomes. Some frequently used functions such as marking a coordinate as a centromere or FISH signal/band, finishing chromosome measurements have both a hotkey and an icon.

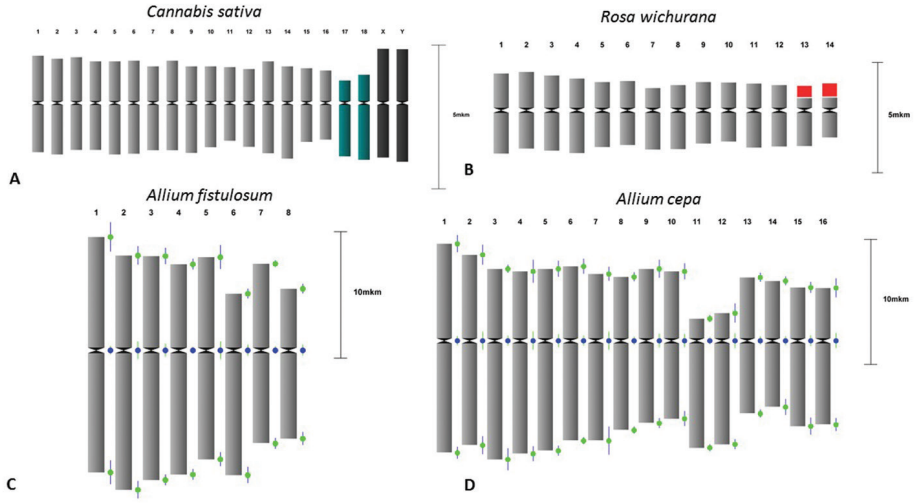

Figure 2. Examples of basic karyotype measurements and idiogram constuction by DRAWID. **A** Chromosomes of *C. sativa* (2*n* = 20); sex (black color) and NOR-bearing (green color) chromosomes are highlighted **B** Idiogram of *Rosa wichurana*; satellites on chromosomes 13 and 14 are colored in red **C** Idiogram of *Allium fistulosum* after measurement of the chromosomes, and application of the function "reduce karyo" merging homologuous chromosomes to obtain a monoploid idiogram, standard deviation bars are shown **D** Idiogram of *Allium cepa* constructed after measurements of 3 metaphases and application of the "get average karyo" function to obtain the average idiogram, standard deviation bars are shown.

The DRAWID program has a dedicated web page (http://www.drawid.xyz) on the website of the Department of plant genetics, biotechnology and breeding of Russian State Agrarian University – MTAA. All the described functions and some other functions are explained there in detail in the manual. In addition, version history and information about reported and solved bugs are published here and will be updated on a regular basis.

Validation

Example 1. Basic karyotyping and averaged idiogram

In order to assess DRAWID for karyotyping of individual metaphases (Figure 2A, B) as well as of a set of metaphases (Figure 2C, D), we used previously published data from karyotyping of *Cannabis sativa* Linnaeus, 1753 (Divashuk et al. 2014), *Rosa wichurana* Crépin, 1888 (Kirov et al. 2016), *Allium cepa* Linnaeus, 1753 and *A. fistulosum* (de Vries and Jongerius 1988, Kirov et al. 2017). All tested species are diploid, $2n = 2x = 20$ (*Cannabis sativa*), $2n = 2x = 14$ (*Rosa wichurana*), $2n = 2x = 16$ (*Allium cepa* and *A. fistulosum*). Idiograms for these species constructed by DRAWID are presented in Figure 2. Our results fully coincided with the data of karyotyping published earlier.

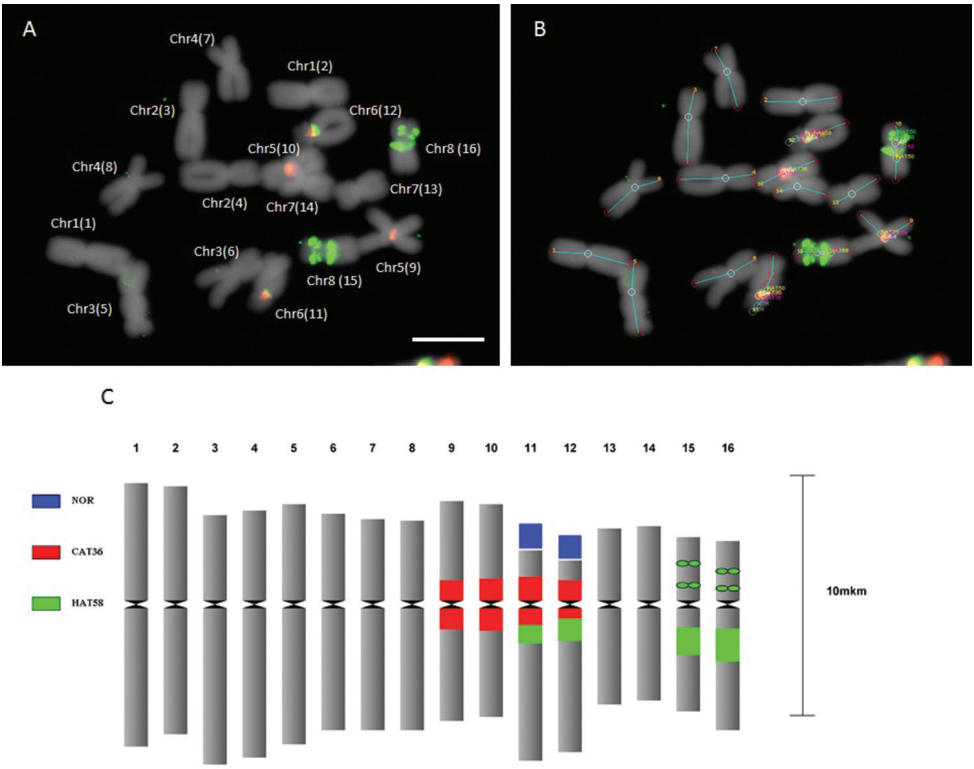

Figure 3. A Metaphase chromosomes of *A. fistulosum* after FISH with HAT58 (green signal) and CAT36 (red signals) repeats. Numbers in brackets correspond to the numbers on the idiogram (**C**) **B** The same picture as in **A** but after measurements by DRAWID **C** Idiogram obtained from the measurements in **A** and **B** Scale bar – 10 μm.

The idiogram can also represent a monoploid chromosome set. For this, DRAWID has a function to convert a diploid or polyploid idiogram into a monoploid one and calculate the mean chromosome index and arm length of homologuous chromosomes. A new idiogram can then be drawn with indication of standard deviation bars. To demonstrate this, measurements of *Allium fistulosum* metaphase and idiogram construction using the function 'reduce karyo' was performed in DRAWID (Figure 2C).

In another example, three metaphases of *Allium cepa* were measured, data were collected into the storage container and an average idiogram was obtained using the "average karyo" function (Figure 2D). Standard deviations of chromosome arm lenghts and centromere indices are presented which provide useful information for the estimation of chromosome parameter variability and comparative analysis.

Example 2. FISH based idiogram

One major added value of DRAWID compared to other software is that it allows to measure FISH signals and indicate them on the idiogram. Figure 3 shows an idiogram

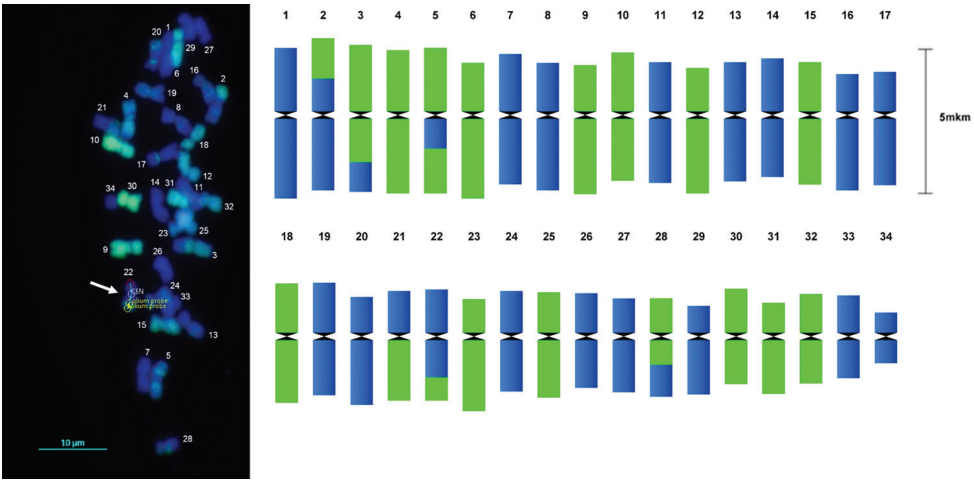

Figure 4. Idiogram (right) obtained from metaphase measurements of an F2 × *Festulolium* hybrid (2*n* = 34) after GISH analysis (left) with *Lolium perenne* genomic DNA labeled as a probe (Dig; green pseudocolor) and *Festuca pratensis* used as block DNA, counterstained with DAPI (blue pseudocolor). DRAWID measurements are shown for the recombinant chromosome 22 (arrow, CEN = centromere). Chromosome numbering is according to chromosome length starting with the largest chromosome.

construction of an *A. cepa* metaphase with HAT58 and CAT36 signals. The position of the signals and size (in case of bands) were calculated. Based on these measurements, the idiogram was build by DRAWID, using the scale bar for calibration, and signal positions of HAT58 and CAT36 rDNA probes were indicated.

Example 3. Idiogram after GISH experiments

GISH is a commonly used tool to study genome composition after interspecific crosses. A correctly drawn idiogram with indication of recombination points is important for result interpretation. We tested DRAWID to build idiograms from F2 hybrids between species of *Lolium* Linnaeus, 1753 and *Festuca* Linnaeus, 1753, having a complex genomic constitution with several recombination points. Using DRAWID, chromosome number, chromosome morphology and GISH signals were determined. On the idiogram, parental composition and sites of recombination are clearly visible (Figure 4).

Conclusion

Modern molecular cytogenetics and cytology requires easy-to-use software for measurements of chromosomes. Exciting advances in FISH technology significantly expanded the boundaries of cytogenetics. FISH is a frequently used tool for plant chromosome identification, monitoring of allien DNA in hybrids, evolutionary studies, physical map construction etc. Here, we present a new software called DRAWID, containing a number of useful functions to make processing of chromosome meas-

urements, FISH signal mapping and preparing of publishable idiograms as easy as possible. DRAWID has a number of advantages compared to previously published programs: 1) simultaneous drawing of idiogram, FISH/GISH/banding signals and measuring; 2) easy adjustment of idiogram color, chromosome position and names, 3) possibility to build average idiograms (with error bars) from collections of single metaphase idiograms. In the future, new functions can be added to further simplify the process of cytogenetic image analysis and idiogram drawing. All updates will be immediately available for the scientific community.

Authors' contributions

IK, LK, KVL wrote the paper and designed the experiments. DR, AS, SM, IF tested and debugged the program. IK designed the webpage.

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RESEARCH ARTICLE

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Chromosomal identification of cryptic species sharing their DNA barcodes: *Polyommatus* (*Agrodiaetus*) *antidolus* and *P.* (*A.*) *morgani* in Iran (Lepidoptera, Lycaenidae)

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Abstract

DNA barcoding has been suggested as a universal tool for molecular species identification; however, it cannot be applied in cases when morphologically similar species share their DNA barcodes due to the common ancestry or mitochondrial introgression. Here we analyze the karyotype of *Polyommatus* (*Agrodiaetus*) *morgani* (Le Cerf, 1909) from the region of its type locality in the southern Zagros Mountains in Iran, provide first chromosomal evidence for *P.* (*A.*) *antidolus* (Rebel, 1901) in Iran and demonstrate that these two species can be easily identified through analysis of their karyotypes whereas they share their mitochondrial barcodes.

Keywords

Ancestral polymorphism, biodiversity, chromosomes, chromosomal fusion/fission, cryptic species, cytogenetics, DNA barcoding, incomplete lineage sorting, karyosystematics, mitochondrial introgression, nomenclature, species identification, taxonomy

Introduction

Cryptic species, morphologically indistinguishable or highly similar biological entities, represent a substantial portion of plant and animal diversity, and therefore the search for these species is important for taxonomic, ecological and evolutionary studies (Beheregaray and Caccone 2007, Pfenninger and Schwenk 2007, Dincă et al. 2013, Vodă et al. 2015). Cryptic species can usually be identified through analysis of molecular markers (Vodă et al. 2015), e.g. through analysis of the so-called DNA barcodes, short genetic sequences from a standard part of the genome (Hebert et al. 2003). However, the use of the standard DNA barcodes such as short fragments of the mitochondrial gene *COI* and the non-coding nuclear sequence, *internal transcribed spacer 2* (*ITS2*), is sometimes insufficient to distinguish between evolutionarily young sister species, either because they can be weakly differentiated regarding these markers or because they are too polymorphic (Avise 2000, Lukhtanov et al. 2015a, b, 2016). The absence of lineage sorting among species can often pose a problem for the use of molecular markers in rapidly evolving taxa because the time to coalescence for alleles within lineages can be greater than the time required for speciation (Avise 2000, Kandul et al. 2004). Chromosomal characters in many groups can evolve more rapidly (Lukhtanov 2015, Vershinina and Lukhtanov 2017), and because they are often present as fixed differences, these characters could serve as applicable markers for recently evolved taxa (King 1993, Dobigny et al. 2005, Lukhtanov et al. 2015a, Vishnevskaya et al. 2016).

Polyommatus (*Agrodiaetus*) *antidolus* (Rebel, 1901), *P.* (*A.*) *kurdistanicus* (Forster, 1961) and *P.* (*A.*) *morgani* (Le Cerf, 1909), a complex of three closely related allopatric species distributed in east Turkey as well as in west and central Iran (Fig. 1) (Eckweiler and Bozano 2016), represent a good example of such situation. Despite morphological similarity (Fig. 2) and identity of *COI* barcodes in the majority of the studied populations (see Table 2 and sequences published in Wiemers 2003, Wiemers and Fiedler 2007, Kandul et al. 2004, 2007, Lukhtanov et al. 2015b and see Lukhtanov et al. 2015b for the exceptions), they can be easily identified by their chromosome numbers. Haploid chromosome numbers (n) were found to be n=25-27 in *P.* (*A.*) *morgani*, n=39-42 in *P.* (*A.*) *antidolus* and n=61-62 in *P.* (*A.*) *kurdistanicus* (de Lesse 1960, 1961, Lukhtanov et al. 1998, 2005, 2015b). However, the karyotype has never been studied in Iranian populations from the southern and northern Zagros Mountains including the region of the type locality of *P.* (*A.*) *morgani* (locality 1 in Fig. 1), and this negatively affects the identification and taxonomic interpretation of all known populations. Here we provide first chromosomal data for populations of the complex from the southern and northern Zagros Mountains.

Material and methods

The butterflies were collected in 2016 in north-west and central Iran: in a mountain valley between Fereydunshahr and Sibak (locality 2), in the vicinity of Darman (25

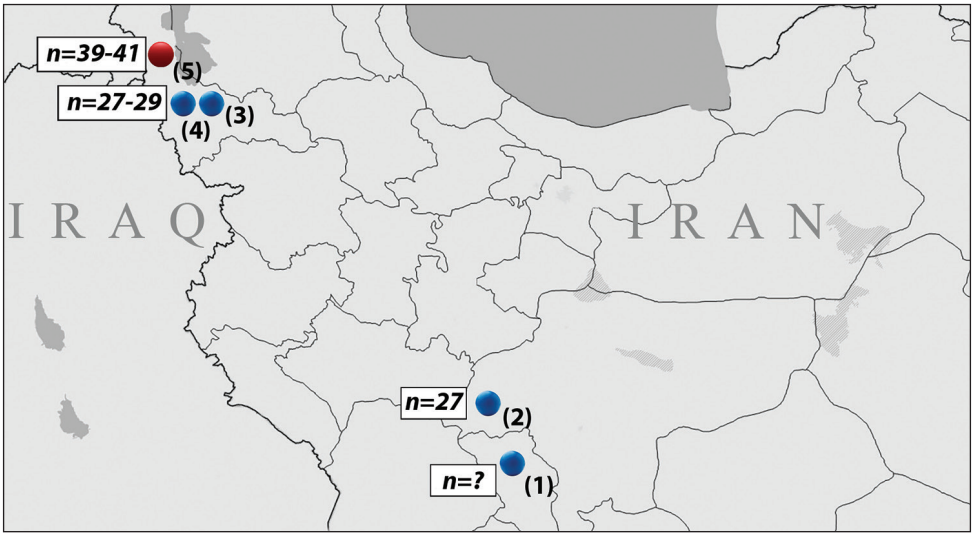

Figure 1. Map of Iran showing the type locality of *Polyommatus* (*Agrodiaetus*) *morgani* and the localities of the analyzed specimens of *P.* (*A.*) *morgani* and *P.* (*A.*) *antidolus*. **1** type locality of *P.* (*A.*) *morgani*, "Deh-Tcheshma" (Deh Cheshme near Farsan, Chaharmahal and Bakhtiari Province) **2** *P.* (*A.*) *morgani*, n=27, vic. Sibak, Esfahan Province **3** *P.* (*A.*) *morgani*, n=27-29, 25 km E of Mahabad, W. Azerbaijan Province **4** *P.* (*A.*) *morgani*, n=27-29, 15 km W of Mahabad, W. Azerbaijan Province **5** *P.* (*A.*) *antidolus*, n=39-41, Seir, 4 km S of Urmia, W. Azerbaijan Province.

km E of Mahabad) (locality 3), in the vicinity of Khalifen (15 km W of Mahabad) (locality 4) and in Seir (near Urmia) (locality 5) (Fig. 1). We also included sequences of karyotyped *P.* (*A.*) *kurdistanicus* and *P.* (*A.*) *antidolus* specimens available from GenBank (Wiemers 2003, Lukhtanov et al. 2005) in our analysis. A complete list of specimens included in this study and information about sampling localities are given in Table 1. Karyotypes (Figs 3 and 4) and *COI*-barcodes (Table 1 and 2) were analyzed using approaches described previously (Lukhtanov et al. 2014, Przybyłowicz et al. 2014). We use the following abbreviations: MI for metaphase I of meiosis and MII for metaphase II of meiosis. Divergences between *COI* sequences were computed using MEGA6 software (Tamura et al. 2013).

Results and discussion

In order to investigate the topotypical population of *P.* (*A.*) *morgani*, we first searched for it in its exact type locality in "Deh Tcheshma" (mountain area near the village Deh Cheshme, close to the city Farsan, Chaharmahal and Bakhtiari Province, Iran) (locality 1 in Fig. 1). Unfortunately, we were unable either to find it there or to locate a biotope suitable for butterflies of the *P.* (*A.*) *antidolus* - *P.* (*A.*) *kurdistanicus* - *P.* (*A.*) *morgani* complex. In our opinion, *P.* (*A.*) *morgani* is extinct in its type locality, probably due to

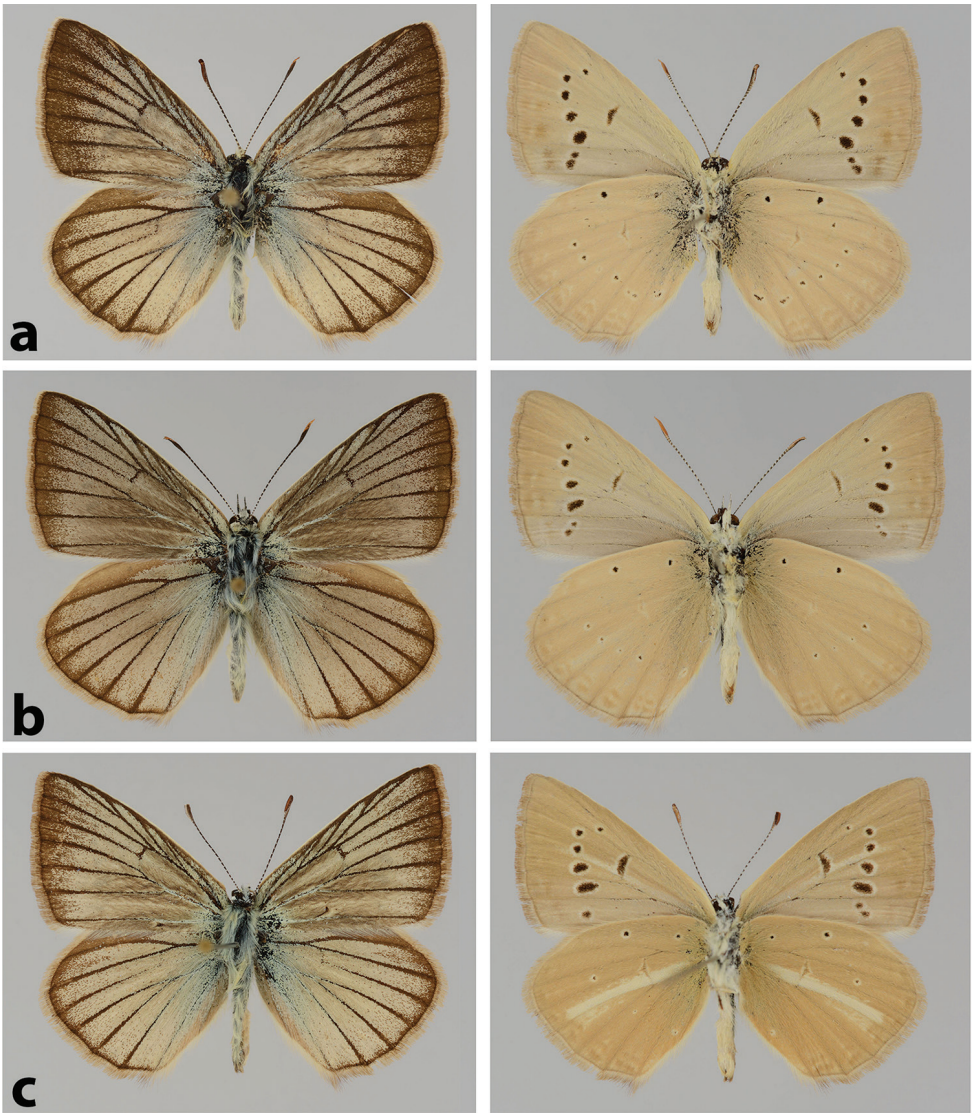

Figure 2. Male wing pattern of *P.* (*A.*) *morgani*, *P.* (*A.*) *antidolus* and *P.* (*A.*) *kurdistanicus.* **a** *P.* (*A.*) *morgani,* Iran, Kordestan Province, Senandaj, 1800 m, 20 July 2000, leg. P. Hofmann **b** *P.* (*A.*) *antidolus* Turkey, Hakkari Province, Ogul-Tal, 1500–1900 m, 1 August 1984, leg. Schurian **c** *P.* (*A.*) *kurdistanicus* Turkey, Van Province, 10 km S of Van, 1900–2100 m, 10 August 1978, leg. Görgner.

climate change and aridification during the last 100 years. Fortunately, we were able to find typical *P.* (*A.*) *morgani* in a small, relatively humid mountain valley between Fereydunshahr and Sibak, 90 km NW of Farsan (N32°55; E50°04', Esfahan Province, Iran) (locality 2 in Fig. 1). In two studied specimens from the latter locality, at the MI stage, the haploid chromosome number n = 27 was found (Figs 3a, b). The meiotic karyotype was strongly asymmetric, with a group of larger bivalents (from 6 to 10 in

Table 1. List of studied material (17 specimens). Asterisks indicate unsequenced specimens. Collectors: E. Pazhenkova (EP), N. Shapoval (NS), V. Lukhtanov (VL).

Field Code	GenBank number	Taxon	Chromosome number (n)	Locality	Altitude	Date	Collectors/ References
Q055*		*morgani* ♂	n=27	Iran, Esfahan Prov., Sibak (N32°55'; E50°04')	2700 m	02.08.2017	EP, NS, VL
Q060*		*morgani* ♂	n=27	Iran, Esfahan Prov., Sibak (N32°55'; E50°04')	2700 m	02.08.2017	EP, NS, VL
Q150	MG457163	*morgani* ♂	n=28-29	Iran, W. Azerbaijan Prov., vic. Darman, 25 km E of Mahabad (N36°45'; E45°52')	1900–2000 m	10.08.2017	EP, NS, VL
Q170	MG457164	*morgani* ♂	n=27	Iran, W. Azerbaijan Prov., vic. Darman, 25 km E of Mahabad (N36°45'; E45°52')	1900–2000 m	10.08.2017	EP, NS, VL
Q171	MG457165	*morgani* ♂	n=27	Iran, W. Azerbaijan Prov., vic. Darman, 25 km E of Mahabad (N36°45'; E45°52')	1900–2000 m	10.08.2017	EP, NS, VL
Q181	MG457166	*morgani* ♂	n=28	Iran, W. Azerbaijan Prov., vic. Darman, 25 km E of Mahabad (N36°45'; E45°52')	1900–2000 m	10.08.2017	EP, NS, VL
Q196	MG457167	*morgani* ♂	n=27-28	Iran, W. Azerbaijan Prov., vic. Khalifen, 15 km W of Mahabad (N36°45'; E45°32')	2100–2200 m	11.08.2017	EP, NS, VL
Q197	MG457168	*morgani* ♂	n=27	Iran, W. Azerbaijan Prov., vic. Khalifen, 15 km W of Mahabad (N36°45'; E45°32')	2100–2200 m	11.08.2017	EP, NS, VL
Q198	MG457169	*morgani* ♂	n=28-29	Iran, W. Azerbaijan Prov., vic. Khalifen, 15 km W of Mahabad (N36°45'; E45°32')	2100–2200 m	11.08.2017	EP, NS, VL
Q237	MG457170	*antidolus* ♂	n=40-41	Iran, W. Azerbaijan Prov., vic. Seir, Urmia (N37°28'; E45°02')	1750 m	14.08.2017	EP, NS, VL
Q238	MG457171	*antidolus* ♂	n=39-40	Iran, W. Azerbaijan Prov., vic. Seir, Urmia (N37°28'; E45°02')	1750 m	14.08.2017	EP, NS, VL
Q239	MG457172	*antidolus* ♂	n=39	Iran, W. Azerbaijan Prov., vic. Seir, Urmia (N37°28'; E45°02')	1750 m	14.08.2017	EP, NS, VL
	AY557093	*antidolus* ♂	n=42	Turkey, Hakkari Prov., Dez Çay	1500 m	22.07.1999	Wiemers 2003
	AY557095	*antidolus* ♂	n=ca44	Turkey, Hakkari Prov., Haruna Geçidi, SE Yüksekova	2000 m	21.07.1999	Wiemers 2003
	AY557108	*kurdistanicus* ♂	n=ca>55	Turkey, Van Prov., Erek Dagi	2200 m	25.07.1999	Wiemers 2003
	AY557074	*kurdistanicus* ♂	n=ca54-56	Turkey, Van Prov., Çatak	1600–1900 m	25.07.1999	Wiemers 2003
	AY496762	*kurdistanicus* ♂	n=62	Turkey, Van Prov., Çatak		July 2001	Lukhtanov et al. 2005

different cells) and a group of smaller bivalents (from 17 to 21 in different cells). The number of bivalents that were classified as "larger" and "smaller" was variable, most likely depending on the bivalent orientation. However, in some metaphase plates, the distinction between the larger and smaller bivalents was unclear, and the bivalents gradually decreased in size, with the largest bivalent approximately 10 times larger than the smallest one. Thus, the results obtained confirm the previous taxonomic interpretations (de Lesse 1960, 1961, Carbonell 2003, Lukhtanov et al. 1998, 2005, 2015b, Eckweiler and Bozano 2016) that considered the populations with n=25–27 as *P.* (*A.*) *morgani*.

Table 2. Divergence between *COI* sequences. The numbers of base differences per site between sequences are shown. The shared barcodes (uncorrected *COI p*-distance = 0) are shown in bold.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
(1) AY557095 *antidolus*															
(2) AY557089 *antidolus*	0.0015														
(3) AY557108 *kurdistanicus*	0.0015	**0**													
(4) AY557074 *kurdistanicus*	0.0015	**0**	**0**												
(5) AY496762 *kurdistanicus*	0.0015	**0**	**0**	**0**											
(6) Q150 *morgani*	0.0015	**0**	**0**	**0**	**0**										
(7) Q170 *morgani*	0.0029	**0**	**0**	**0**	**0**	**0**									
(8) Q171 *morgani*	0.0029	0.0015	0.0015	0.0015	0.0015	0.0015	0.0015								
(9) Q181 *morgani*	0.0015	**0**	**0**	**0**	**0**	**0**	**0**	0.0015							
(10) Q196 *morgani*	0.0015	**0**	**0**	**0**	**0**	**0**	**0**	0.0015	**0**						
(11) Q197 *morgani*	0.0015	**0**	**0**	**0**	**0**	**0**	**0**	0.0015	**0**	**0**					
(12) Q198 *morgani*	0.0015	**0**	**0**	**0**	**0**	**0**	**0**	0.0015	**0**	**0**	**0**				
(13) Q237 *antidolus*	0.0015	**0**	**0**	**0**	**0**	**0**	**0**	0.0015	**0**	**0**	**0**	**0**			
(14) Q238 *antidolus*	0.0015	**0**	**0**	**0**	**0**	**0**	**0**	0.0015	**0**	**0**	**0**	**0**	**0**		
(15) Q239 *antidolus*	0.0015	**0**	**0**	**0**	**0**	**0**	**0**	0.0015	**0**	**0**	**0**	**0**	**0**	**0**	

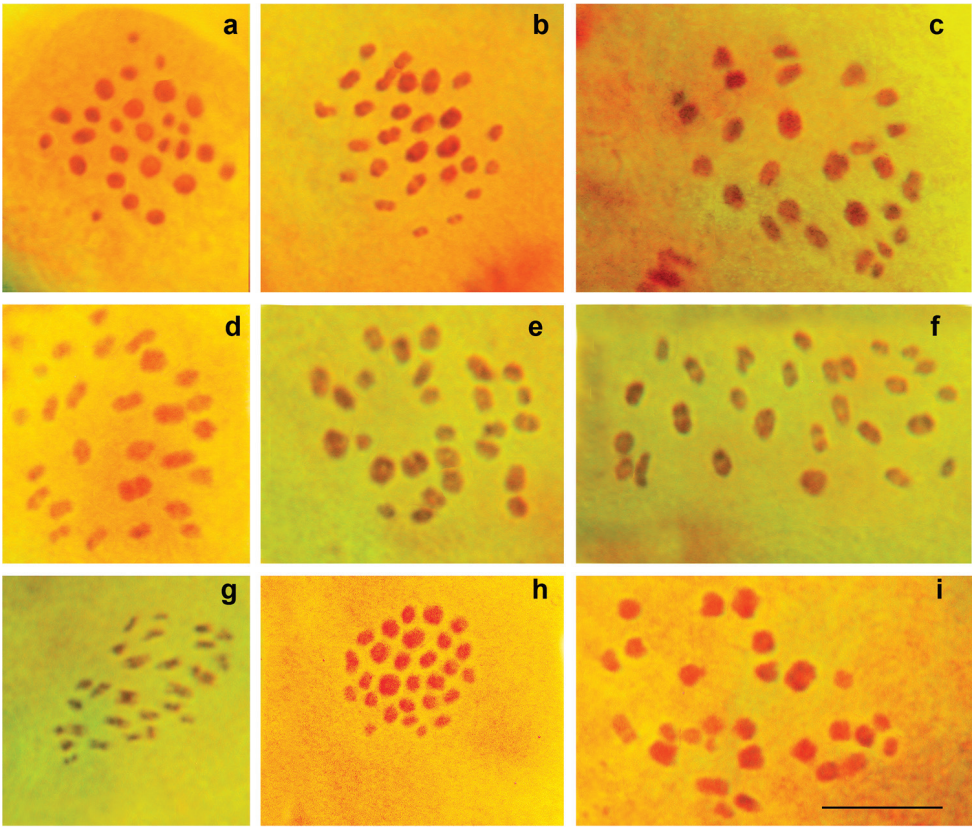

Figure 3. Karyotype of *P.* (*A.*) *morgani* **a** Q060, MI, n=27 **b** Q055, MI, n=27 **c** Q170, prometaphase I, n=27 **d** Q171, MI, n=27 **e** Q197, prometaphase I, n=27 **f** Q196, MI, n=27 **g** Q196, MII, n=27 **h** Q196, MII, n=28 **i** Q196, MII, n=28. Bar = 10 μ.

Tshikolovets et al. (2014) and Eckweiler and Bozano (2016) identified the population of the *P.* (*A.*) *antidolus* - *P.* (*A.*) *kurdistanicus* - *P.* (*A.*) *morgani* complex from the vicinity of Mahabad (West Azerbaijan Province) (localities 3 and 4 in Fig. 1) as *P.* (*A.*) *antidolus*; however, they did not provide any chromosomal data to confirm this conclusion. We analyzed seven specimens from two localities close to Mahabad (localities 3 and 4 in Fig. 1). At the prometaphase I, MI and MII stages, n=27 was determined as the basic number in four specimens (Fig. 3c–g), not n=39-42 as expected for *P.* (*A.*) *antidolus*. The number of elements within the karyotype was unstable, varying from n=27 to n=29, most likely due to the presence of two chromosomal fusions/fissions (Figs 3h, i, 4a, b). With respect to the karyotype structure (size and proportion of larger vs. smaller chromosomal elements) the specimens from Mahabad were indistinguishable from the typical *P.* (*A.*) *morgani* described above. The chromosome numbers n=28 and n=29 were not previously reported for *P.* (*A.*) *morgani* (de Lesse 1960, 1961, Lukhtanov et al. 1998, 2005, 2015b). However, since there is no fixed chromosomal difference between the popula-

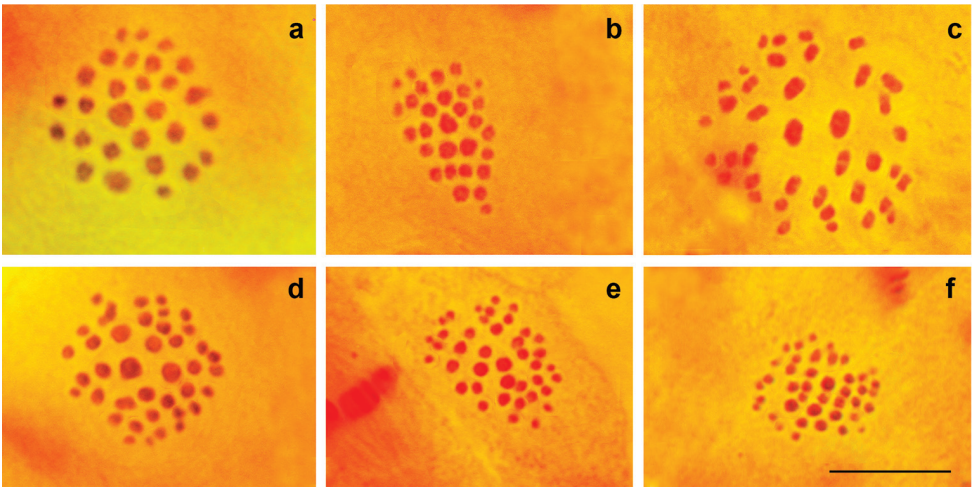

Figure 4. Karyotypes of *P.* (*A.*) *morgani* and *P.* (*A.*) *antidolus*. **a** *P.* (*A.*) *morgani*, Q150, MII, n=28 **b** *P.* (*A.*) *morgani*, Q150, MII, n=29 **c** *P.* (*A.*) *antidolus*, Q239, MI, n=39, squash preparation **d** *P.* (*A.*) *antidolus*, Q239, MI, n=39, intact metaphase plate **e** *P.* (*A.*) *antidolus*, Q237, MII, n=40 **f** *P.* (*A.*) *antidolus*, Q237, MII, n=41. Scale bar = 10 μ.

tions from Sibak and Mahabad, we do not see the need for a description of a new taxon from Mahabad, and therefore identify the populations from Mahabad as *P.* (*A.*) *morgani*.

Finally, in three specimens collected in Seir (near Urmia, locality 5 in Fig. 1) at the MI/MII stages, we found that the number of chromosomal elements varied from 39 to 41. The chromosomes ranged in size from very small to large (Fig. 4c–f). This karyotype (n=39-41) seems to be identical to that found in *P.* (*A.*) *antidolus* in the neighboring Province Hakkari in south-east Turkey, thus providing first chromosomal evidence for *P.* (*A.*) *antidolus* in Iran.

Acknowledgements

We are grateful to Wolfgang ten Hagen for providing information about sampling localities of *P.* (*A.*) *morgani* in Iran. The complete financial support for this study was provided by the grant from the Russian Science Foundation N 14-14-00541 to The Zoological Institute of the Russian Academy of Sciences. The work was partially performed using equipment of the Centre for Molecular and Cell Technologies and Department of Entomology of St. Petersburg State University. A part of this equipment was purchased with support of the St. Petersburg University grant 1.40.490.2017.

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RESEARCH ARTICLE

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A new butterfly species from south Russia revealed through chromosomal and molecular analysis of the *Polyommatus* (*Agrodiaetus*) *damonides* complex (Lepidoptera, Lycaenidae)

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Abstract

Finding a new species is a rare event in easy-to-see and well-studied organisms like butterflies, especially if they inhabit well-explored areas such as the Western Palaearctic. However, even in this region, gaps in taxonomic knowledge still exist and here we report such a discovery. Using a combined analysis of chromosomal and molecular markers we demonstrate that *Polyommatus* blue populations from Daghestan (South Russia), previously identified as *P. aserbeidschanus*, represent in fact a new species which is described here as *P. australorossicus* **sp. n.** We also show that the enigmatic *Polyommatus damonides* described as a form of *Polyommatus damone* and later considered as an entity similar to *P. poseidon* or *P. ninae* is conspecific with a taxon previously known as *P. elbursicus*. As a result of our study, we propose several taxonomic changes within the *P. damonides* species complex and suggest the following new combinations: *P. damonides elbursicus* Forster, 1956, **comb. n.** and *P. damonides gilanensis* Eckweiler, 2002, **comb. n.**

Keywords

Ancestral polymorphism, biodiversity, chromosomes, chromosomal fusion/fission, *COI*, cryptic species, DNA barcoding, incomplete lineage sorting, inverted meiosis, karyosystematics, molecular phylogenetics, mitochondrial introgression, phylogeography, speciation

Introduction

Agrodiaetus Hübner, 1822, a subgenus of the species-rich Palaearctic genus *Polyommatus* Latreille, 1804 (Talavera et al. 2013), includes numerous species, subspecies and forms with uncertain taxonomic positions (de Lesse 1960a, b, Eckweiler and Häuser 1997, Häuser and Eckweiler 1997, Olivier et al. 1999, Carbonell 2000, 2001, Dantchenko 2000a, Przybyłowicz 2000, ten Hagen and Eckweiler 2001, Skala 2001, Lukhtanov and Dantchenko 2002a, b, Kandul et al. 2004, Wiemers 2003, Schurian and ten Hagen 2003, Vila et al. 2010, Talavera et al. 2013, Eckweiler and Bozano 2016). It was estimated to have originated only about 3 million years ago (Kandul et al. 2004) and radiated rapidly in the Western Palaearctic (Kandul et al. 2007). The last published review of the subgenus includes 120 valid species (Eckweiler and Bozano 2016). Many of them have extremely local 'dot-like' distributions that are restricted to particular mountain valleys in the Balkan Peninsula, Asia Minor, Transcaucasus, Iran and Central Asia (Vila et al. 2010, Lukhtanov et al. 2015a,b, Eckweiler and Bozano 2016, Vishnevskaya et al. 2016). This subgenus is a model system in studies of speciation (Lukhtanov et al. 2005, Wiemers et al. 2009), intraspecific differentiation (Dincă et al. 2013, Przybyłowicz et al. 2014), and rapid karyotype evolution (Lukhtanov and Dantchenko 2002a, Kandul et al. 2007, Vershinina and Lukhtanov 2013, 2017).

Species identification in *Agrodiaetus* is complicated. The morphology of male genitalia is uniform for most of the species. With a few exceptions, it can help to separate groups of species (Coutsis 1986), but not individual species. The differences in wing pattern and coloration (Eckweiler and Bozano 2016) as well as in the number of antennal segments (Carbonell 1993) are very subtle or nearly lacking between many *Agrodiaetus* species. The specific pubescence of costal area of forewings may be a useful morphological character to separate species in syntopy (Dantchenko and Churkin 2003), but it works only in certain cases. In spite of morphological similarity, the taxonomic and identification problems within the subgenus *Agrodiaetus* can be solved if chromosomal (de Lesse 1960a,b, Lukhtanov 1989) or molecular markers (Wiemers 2003, Kandul et al. 2004, 2007, Lukhtanov et al. 2005, Stradomsky and Fomina 2013), or their combination (Lukhtanov et al. 2006, 2008, 2014, 2015a,b, Vila et al. 2010, Lukhtanov and Tikhonov 2015, Shapoval and Lukhtanov 2015a, b) are applied. An unusual diversity of karyotypes is the most remarkable characteristic of *Agrodiaetus*. Species of this subgenus exhibit one of the highest ranges in chromosome numbers in the animal kingdom (Lukhtanov 2015). Haploid chromosome numbers (n) in *Agrodiaetus* range from n=10 in *P.* (*A.*) *caeruleus* (Staudinger, 1871) to n=134 in *P.* (*A.*) *shahrami* (Skala, 2001) (Lukhtanov and Dantchenko 2002a, Lukhtanov et al. 2005). Additionally, this subgenus demonstrates a high level of karyotypic differentiation with respect to chromosome size (Lukhtanov and Dantchenko 2002b) and variation in number of chromosomes bearing ribosomal DNA clusters (Vershinina et al. 2015). These differences provide reliable characters for species delimitation, description and identification (de Lesse 1960a, b, Lukhtanov and Dantchenko 2002a, b).

Here we use a combination of molecular mitochondrial (*COI*) and nuclear chromosomal (karyotype) markers to analyze the taxa and populations close to *Polyommatus damonides* (= lineage VIII in Kandul et al. 2004). This group includes the following species: *P. ninae* (Forster, 1956), *P. aserbeidschanus* (Forster, 1956), *P. australorossicus* sp. n., *P. damonides* (Staudinger, 1899), *P. lukhtanovi* (Dantchenko, 2005), *P. zarathustra* Eckweiler, 1997, *P. arasbarani* (Carbonell & Naderi, 2000) and *P. pierceae* (Lukhtanov & Dantchenko, 2002). Here we do not analyze the distantly related taxa *P. paulae* Wiemers & De Prins J., 2004, *P. huberti* (Carbonell, 1993), *P. turcicolus* (Koçak, 1977), *P. zapvadi* (Carbonell, 1993), *P. avajicus* (Blom, 1979) and *P. zardensis* Schurian & ten Hagen, 2001 which will be considered in later publications. The taxa of the *P. damonides* species complex were revised by Forster (1956, 1960, 1961), de Lesse (1963), Lukhtanov (1989), Carbonell (1993), Hesselbarth et al. (1995), Carbonell and Naderi (2000), Dantchenko (2000b, 2005) and Eckweiler and Bozano (2016). However, the species-level boundaries remain poorly defined in this complex.

Material and methods

Samples

Specimens examined (Supplementary Table 1, Fig. 1) are deposited in the Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia and in the McGuire Center for Lepidoptera and Biodiversity (MGCL), Florida Museum of Natural History, University of Florida, Gainesville, Florida, USA.

Chromosomal analysis

Karyotypes were obtained from 157 adult males representing eight species and were processed as previously described (Lukhtanov et al. 2014, 2015a, Vishnevskaya et al. 2016). Briefly, gonads were removed from the abdomen and placed into freshly prepared fixative (3:1; 96% ethanol and glacial acetic acid) directly after capturing the butterfly in the field. Testes were stored in the fixative for 3-36 months at +4°C. Then the gonads were stained in 2% acetic orcein for 30-60 days at +18-20°C. Different stages of male meiosis, including metaphase I (MI) and metaphase II (MII) were examined using an original two-phase method of chromosome analysis (Lukhtanov and Dantchenko 2002, Lukhtanov et al. 2006). In some cases diploid chromosome numbers (2n) were counted in atypical meiosis (see Lorković 1990 for a review of atypical meiosis in Lepidoptera). Abbreviation *ca* (circa) means that the count was made with an approximation due to an insufficient quality of preparation or overlapping of some chromosomes or bivalents.

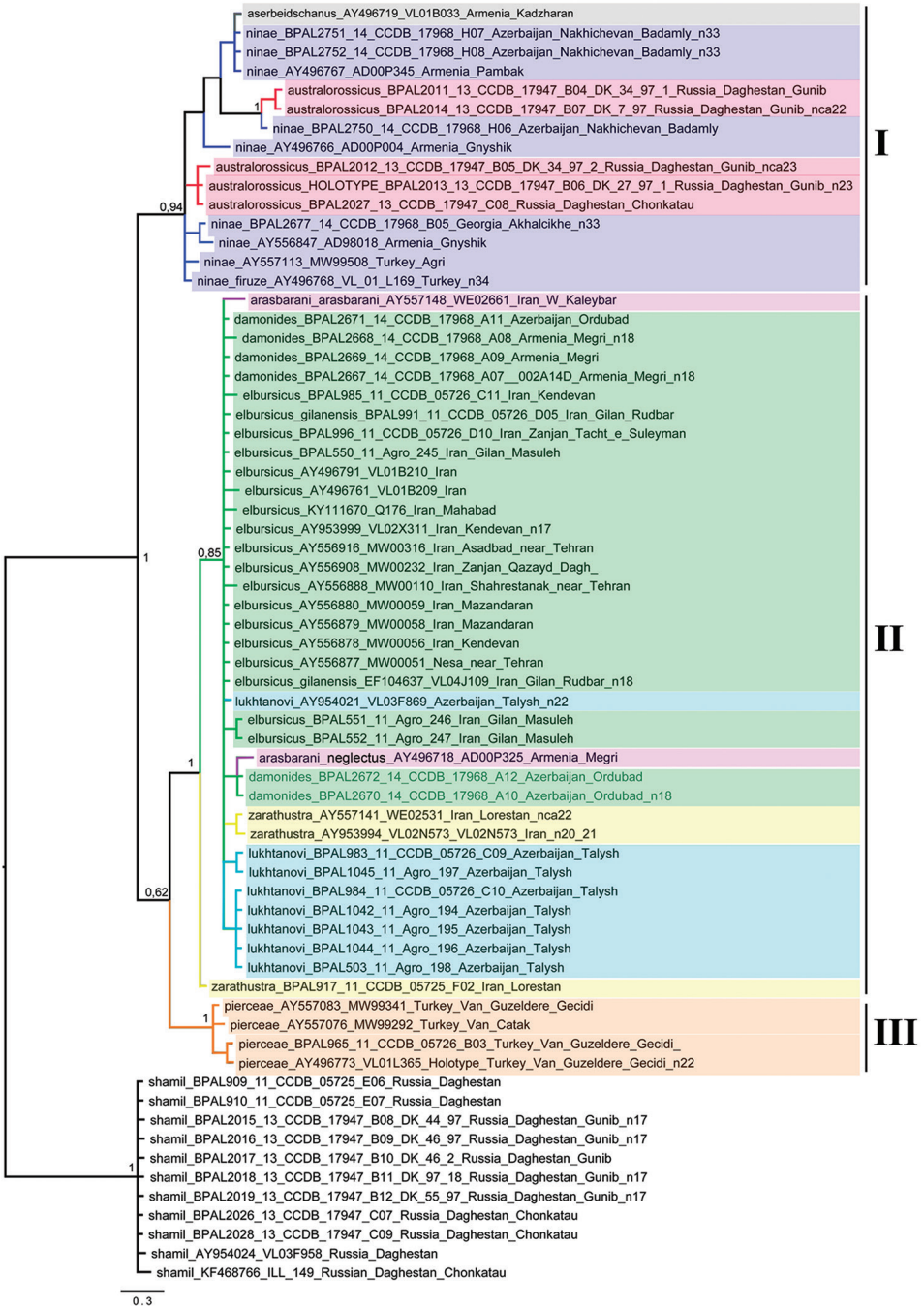

Figure 1. The Bayesian tree of studied *Polyommatus* samples based on analysis of the *cytochrome oxidase subunit I* (*COI*) gene. Numbers at nodes indicate Bayesian posterior probability. I, II and III are recovered haplogroups of the *P. damonides* species complex. *Polyommatus shamil*, phenotypically similar to *P. australorossicus*, but genetically distant, was used to root the tree.

Molecular methods and DNA barcode-based phylogeographic study

Standard *COI* barcodes (658-bp 5' segment of mitochondrial cytochrome oxidase subunit I) were studied. *COI* sequences were obtained from 30 specimens representing the *P. damonides* species group and from 9 samples of *P. shamil* (Dantchenko, 2000) which was selected as outgroup. Legs were sampled from these specimens, and sequence data from the DNA barcode region of *COI* were obtained at the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph) using protocols described in Hajibabaei et al. (2005), Ivanova et al. (2006) and deWaard et al. (2008). Photographs of these specimens, as well as collecting data are available in the Barcode of Life Data System (BOLD), project Butterflies of Palearctic (BPAL) at http://www.boldsystems.org/. Field codes and collecting data of these samples are also shown in Figure 1.

We also used 28 published *COI* sequences (Wiemers 2003, Kandul et al. 2004, Lukhtanov et al. 2005, Kandul et al. 2007, Wiemers and Fiedler 2007, Shapoval and Lukhtanov 2016) which were downloaded from GenBank. Their accession numbers are shown in Figure 1.

The barcode analysis involved 67 *COI* sequences. Sequences were aligned using the BioEdit software (Hall 1999) and edited manually. Phylogenetic hypotheses were inferred using Bayesian inference as described previously (Vershinina and Lukhtanov 2010, Lukhtanov et al. 2016a, b). Briefly, the Bayesian analysis was performed using the program MrBayes 3.2 (Ronquist et al. 2012) with default settings as suggested by Mesquite (Maddison and Maddison 2015): burn-in=0.25, nst=6 (GTR + I + G). Two runs of 10,000,000 generations with four chains (one cold and three heated) were performed. The consensus of the obtained trees was visualised using FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Karyotypes

157 specimens were karyotyped (Supplementary Table 1, Figs 2–7).

P. ninae (Fig. 2a–e)
At the MI/MII stages, the number of chromosome elements was found to vary from n=ca32 to n=34-36 in 21 studied specimens from different localities, with n=33 and n=34 as distinct modal numbers. All chromosome elements formed a gradient size row. The species seemed to be polymorphic for at least one chromosomal fusion/fission resulting in specimens possessing 33 bivalents (homozygotes for fused chromosomes) (Fig. 2b), 32 bivalents + 1 trivalents (heterozygotes for fusion/fission) (Fig. 2c, d) and 34 bivalents (homozygotes for unfused chromosomes) (Fig. 2a). Chromosomal rearrangements involved in formation of karyotypes with higher chromosome number (n=33-35 and n=34-36) remain still unknown.

P. aserbeidschanus (Fig. 2f–h)
At the MI/MII stages, the number of chromosome elements was found to vary from n=32 to n=37 in 17 studied specimens from different localities, with n=33 as a modal number. MI/MII metaphases consisted of elements of progressively decreasing size.

P. australorossicus sp. n. (Fig. 2i)
At the MI/MII stages, the haploid chromosome number n = 23 was found in 6 studied individuals. Elements were found to form a gradient size row in which the largest element was approximately 5 times larger than the smallest element. In two specimens, the diploid chromosome number was estimated as 2n = 46 in male atypical meiosis. In the sample DK-7-97 we counted approximately n = ca22 and in the sample from Chonkatau we counted approximately n = ca24 at the MI stage. The last two counts were done with an approximation due to the overlapping of some bivalents, therefore interpretation of these deviating numbers (a real variation or a mistake of counting) is difficult.

P. damonides damonides from Azerbaijan and Armenia (Fig. 3a–d)
At the MI/MII stages, the haploid chromosome number n = 18 was found in 10 studied individuals. Elements formed a gradient size row in which the largest element was approximately 2-2.5 times larger than the smallest element. In two specimens, the diploid chromosome number was determined as 2n = 36 in male atypical meiosis.

P. damonides from Iran (previously known as *P. elbursicus*) (Fig. 4a–h)
At the MI/MII stages, the haploid chromosome number n = 18 was found in 26 studied individuals. Elements constituted a gradient size row in which the largest element was approximately 2-2.5 times larger than the smallest element. In 7 specimens, the diploid chromosome number was determined as 2n = 36 in male atypical meiosis. Thus, the karyotype of these samples from Iran is indistinguishable from the karyotype of the samples of *P. damonides* from Azerbaijan and Armenia.

P. damonides elbursicus (Forster, 1956) (Fig. 5a–e)
At the MI/MII stages, the haploid chromosome number n = 17 was found in four studied individuals. Elements formed a gradient size row in which the largest element was approximately 2-2.5 times larger than the smallest element. In the sample VL311, the diploid chromosome number was determined as 2n = 34 in male atypical meiosis.

P. damonides gilanensis Eckweiler, 2002 (Fig. 6a–f)
At the MI/MII stages, the number of chromosome elements was found to vary from n=18 to n=19 in three studied specimens collected in the type-locality of this taxon. Elements formed a gradient size row in which the largest element was approximately 2-2.5 times larger than the smallest element. The population was found to be polymorphic for a chromosomal fusion/fission resulting in specimens possessing 18 bivalents (homozygotes for fused chromosomes), 17 bivalents + 1 trivalents (heterozygotes for fusion/fission) (Fig. 6a, b) and 19 bivalents (homozygotes for unfused chromosomes)

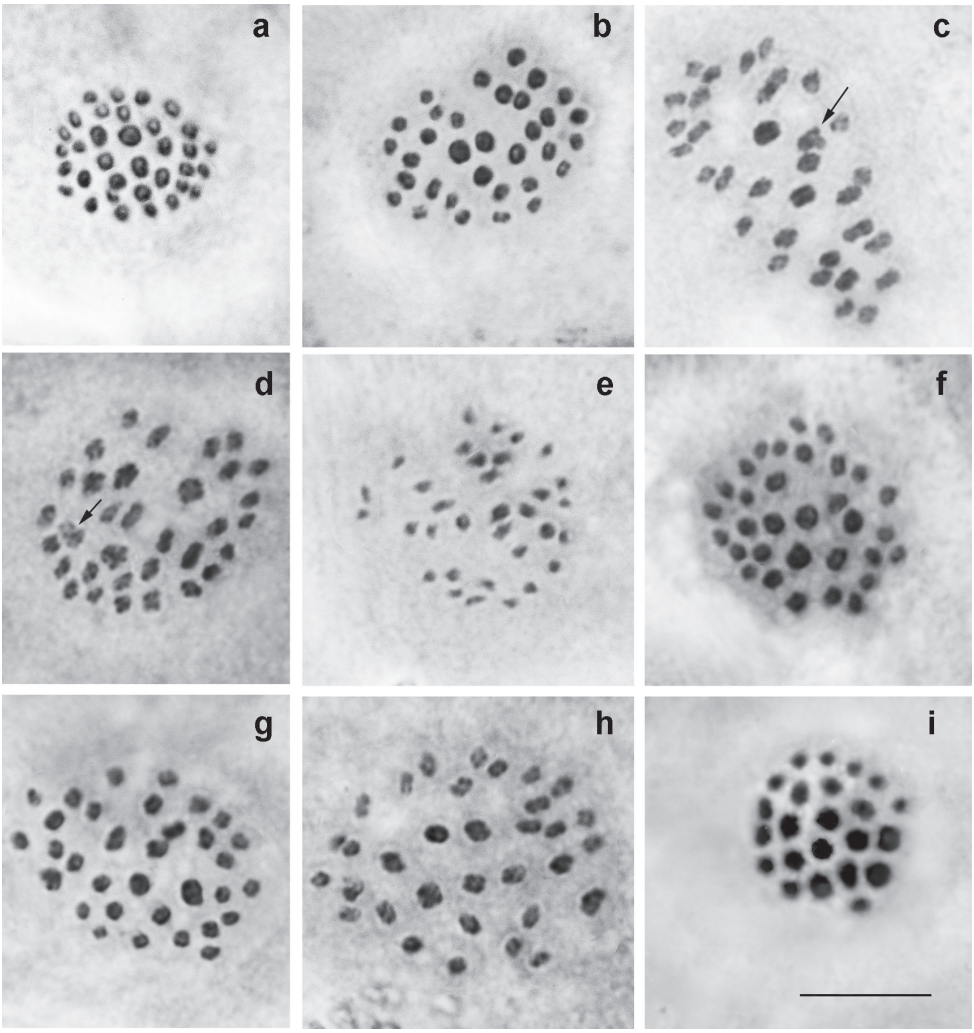

Figure 2. Karyotypes of *P. ninae*, *P. aserbeidschanus* and *P. australorossicus* sp. n. Trivalents are indicated by arrows. **a** *P. ninae*, sample 2014VL34, MI, n=34 **b** *P. ninae*, sample 2014VL39, MI, n=33 **c** *P. ninae*, sample 2014VL33, MI, n=32 bivalents + 1 trivalent (heterozygote for fusion/fission) **d** *P. ninae*, sample 2014VL60, n=32 bivalents + 1 trivalent (heterozygote for fusion/fission) **e** *P. ninae*, sample 2014VL33, MII, n=33 **f** *P. aserbeidschanus*, sample 05A406, MI, n=32 **g** *P. aserbeidschanus*, sample 05A387, MI, n=33 **h** *P. aserbeidschanus*, sample 05A387, MI, n=33 **i** *P. australorossicus* sp. n., sample DK-27-97-1, MI, n=23. Bar = 10 μ.

(Fig. 6d–f). Interestingly, in the case of heterozygocity for fusion/fission, the same number of chromosome elements (n=18) was found at the MI and MII stages, and the trivalent chromosomes (triple chromatids) were observed at both MI and MII stages (Fig. 6a–c).

P. zarathustra (Fig. 7a)
At the MI/MII stages, the number of chromosome elements was found to vary from n=20-21 to n=24 in 6 studied specimens from different localities, with n=22 as a modal number. Elements formed a gradient size row in which the largest element was approximately 5 times larger than the smallest element. The species seemed to be polymorphic for several, still unrecognized chromosomal rearrangements resulting in chromosome number variation.

P. arasbarani arasbarani (Fig. 7b)
At the MI/MII stages, the number of chromosome elements was found to vary from n=23-24 to n=25 in 6 studied specimens, most likely due to polymorphism for a single chromosomal fusion/fission. Elements formed a gradient size row in which the largest element was approximately 5-6 times larger than the smallest element.

P. arasbarani neglectus Dantchenko, 2000 (Fig. 7c)
At the MI stage, the number of chromosome elements was determined to be n=25 in the sample B447. In the samples KA-95-99, 2001-Q456 and 2001-Q457 the number of elements was estimated with an approximation as n=24-25 and n=25-26 due to the overlapping of some bivalents. In the sample KA-98-99, the diploid chromosome number was estimated as 2n = ca 48. Elements formed a gradient size row in which the largest element was approximately 5-6 times larger than the smallest element.

P. lukhtanovi (Fig. 7d, e)
At the MI/MII stages, in 28 of 33 studied specimens the haploid chromosome number was determined as n=22. In one of these 28 specimens atypical meiosis displayed 2n=44. In 3 of 33 studied samples the haploid chromosome number was determined as n=21, and in two samples intraindividual variation in the number of elements was observed: n=21-22. We interpret this result as an evidence for polymorphism for a single fusion/fission resulting in in specimens possessing n=21 (Fig. 7e) and n=21-22 (homozygotes for fusion and heterozygotes for fusion/fission) and n=22 bivalents (homozygotes for the unfused chromosomes) (Fig. 7). Elements formed a gradient size row in which the largest element was approximately 3 times larger than the smallest element.

P. shamil (Fig. 7f)
At the MI/MII stages, in all 12 studied specimens the haploid chromosome number was determined as n=17. In three of these 12 specimens atypical meiosis displayed 2n=34. Elements formed a gradient size row in which the largest element was approximately 2 times larger than the smallest element.

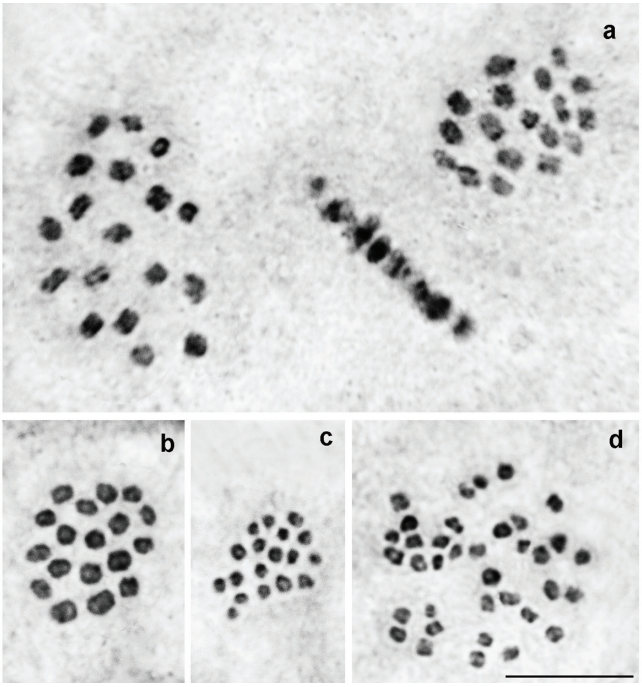

Figure 3. Karyotype of *P. damonides* from Ordubad vicinity in Nakhchivan (Azerbaijan) and Meghri vicinity in Armenia. **a** sample 005A14K, three MI plates (two from polar view and one from equatorial view) displaying n=18 **b** sample 005A14K, MI, n=18 **c** sample 005A14K, MII, n=18 **d** sample 2014VL04, male atypical meiosis, 2n=36. Bar = 10 μ.

COI barcode analysis

The *COI* barcode analysis revealed three major, highly supported clusters within the studied samples (Fig. 1). The first cluster (haplogroup I) is represented by samples of *P. ninae*, *P. aserbeidschanus* and *P. australorossicus*. This cluster inhabits the northern part of the *P. damonides* complex distribution range: the Russian part of the eastern Caucasus (Daghestan), Georgia, Armenia (except its south-eastern part near Meghri), Nakhchivan in Azerbaijan (except Ordubad district) and north-eastern Turkey (Fig. 8).

Within its distribution range *P. ninae* demonstrates a diversity of *COI* haplotypes; however, no distinct intraspecific sublineages were discovered. *Polyommatus ninae firuze* (Carbonell, 1993) described from Turkey (Gümüşhane, Kelkit) shows no differentiation from topotypical populations from Armenia, and in our opinion should be considered no more than a synonym of *P. ninae*.

The specimens of *P. aserbeidschanus* collected in the type locality of this taxon "Armenia, mts. Zangezur, pag. Kadzharantz, pr. Mts. Kapudzhich" (Forster 1956) were found to share their *COI* haplotype with the samples of *P. ninae* from Armenia and Azerbaijan.

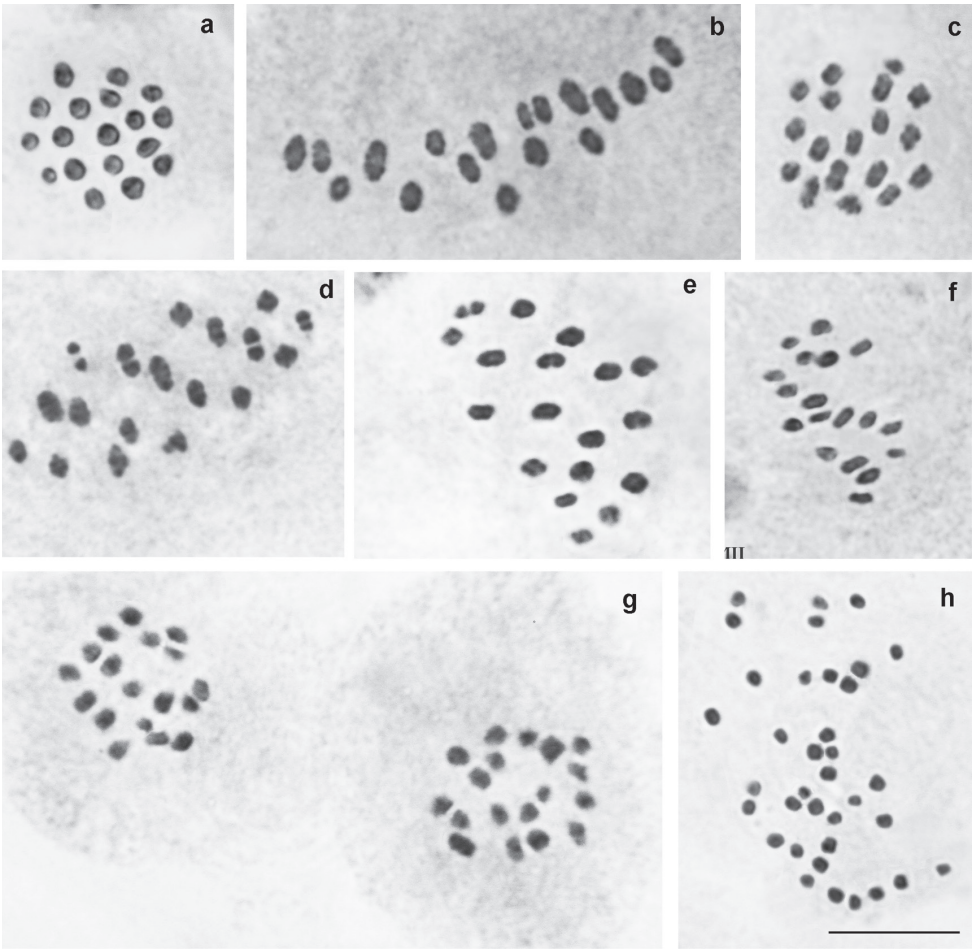

Figure 4. Karyotype of *P. damonides* from Iran (previously known as *P. elbursicus*). **a** sample E234, MI, n=18 **b** sample E193, MI, n=18 **c** sample E460, MI, n=18 **d** sample E237, MI, n=18 **e** sample E459, MI, n=18 **f** sample E193, MII, n=18 **g** sample J573, two sister MII plates, n=18 **h** sample E234, male atypical meiosis, 2n=36. Bar = 10 μ.

On the tree obtained, the samples of *P. australorossicus* were intermixed together with the samples of *P. ninae*. Although no shared haplotypes were found, the uncorrected *p*-distances between the *P. ninae* and *P. australorossicus* samples were quite low varying from 0.2 % to 1.4 %. Thus, both *P. ninae* and *P. australorossicus* appeared on the tree as genetically undifferentiated, non-monophyletic assemblages.

The second lineage (haplogroup II) is represented by samples of *P. damonides*, *P. elbursicus*, *P. elbursicus gilanensis*, *P. zarathustra*, *P. arasbarani* and *P. lukhtanovi*. This is the southern lineage of the *P. damonides* complex distributed in the south-eastern part of Nakhchivan (Ordubad district, Azerbaijan), south-eastern part of Armenia (Meghri district), south-eastern part of Azerbaijan (Talysh) and Iran (Fig. 8). On the tree ob-

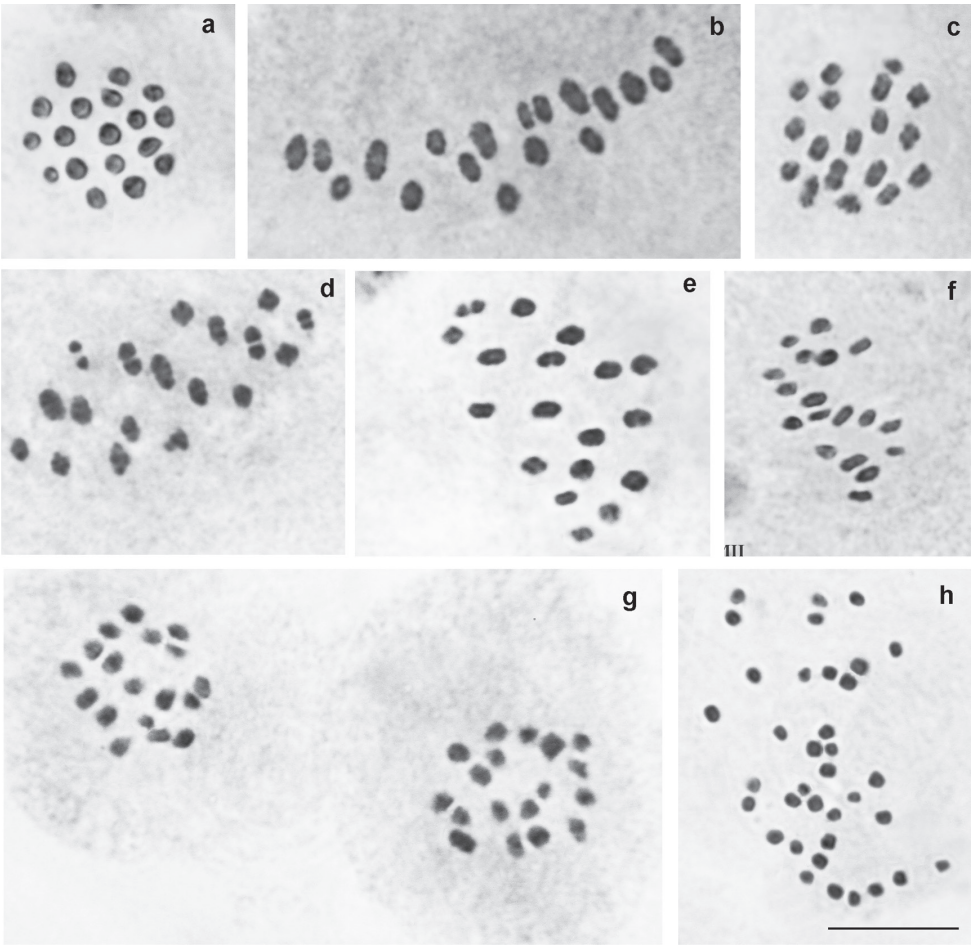

Figure 5. Karyotype of *P. damonides elbursicus* from Elburs Mts (north Iran). **a** sample M804, MI, n=17 **b** sample VL302, two MI plates, each displaying n=17 **c** sample VL302, diakinesis – early prometaphase, n=17 **d** sample VL302, prometaphase, n=17 **e** sample VL302, MII, n=17. Bar = 10 μ.

tained, the samples of this lineage were deeply intermixed, and all these taxa appeared as undifferentiated non-monophyletic assemblages.

The third lineage (haplogroup III) (south-western group) is represented by samples of *P. pierceae*. This lineage inhabits the south–eastern part of Turkey (Fig. 8).

Discussion

Rapid chromosomal evolution and possibility of chromosomal speciation

The *P. damonides* species complex demonstrates a high rate of karyotype evolution resulting in a great interspecific diversity of chromosome numbers (from n=17 to n 34-

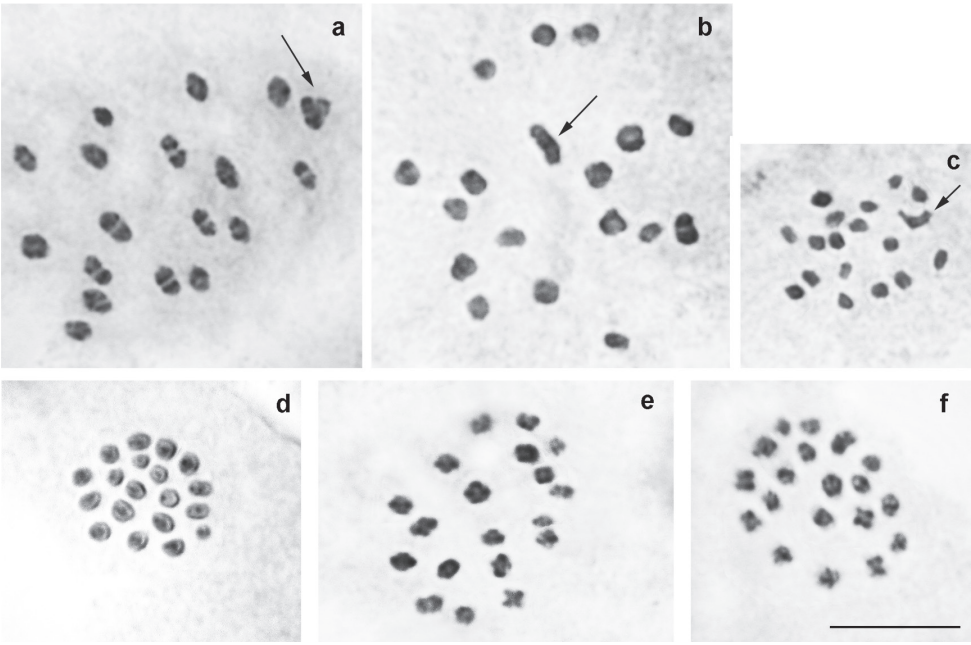

Figure 6. Karyotype of *P. damonides gilanensis* from its type-locality. Trivalents and triple chromatid are indicated by arrows. **a** sample J111, MI, n=18 **b** sample J111, MI, n=18 **c** sample J111, MII, n=18 **d** sample J112, MI, n=19 **e** sample J112, MI, n=19 f sample J112, MI, n=19. Bar = 10 μ.

36) (Figs 2–7) despite the low (between the haplogroups I and II) or lack of (within the haplogroups I and II) (Fig. 1) *COI* differentiation. Fusions and fissions of chromosomes are the most probable rearrangements driving the chromosome number change in the *P. damonides* complex as well as in other butterfly species (Lukhtanov et al. 2011, Šíchová et al. 2015, 2016). In *P. ninae*, *P. damonides gilanensis*, *P. arasbarani* and *P. lukhtanovi* some of these fusions/fissions are found in heterozygous conditions resulting in trivalent formation at the MI stage.

There are two possible ways of the first meiotic division in a cell with the fusion/fission trivalent: (i) resulting in a daughter cell containing two smaller chromosomes and a daughter cell containing one larger chromosome, and (ii) resulting in two daughter cells, each containing an element consisting of three triple chromatids (Nokkala et al. 2006). In case of chiasmate meiosis, Nokkala et al. (2006) interpreted both ways as two different variants of conventional pre-reductional meiosis, whereas Banno et al. (1995) interpreted the second way as post-reductional meiosis. The latter is also known as "inverted meiosis" (see e.g. Heckman et al. 2014, Manicardi et al. 2015, Bogdanov 2016). Despite the difference in the interpretation (in fact despite the difference in the definition of conventional and inverted meiosis), both papers stress the significant distinction between the first and the second ways. In *P. damonides gilanensis* the trivalent-

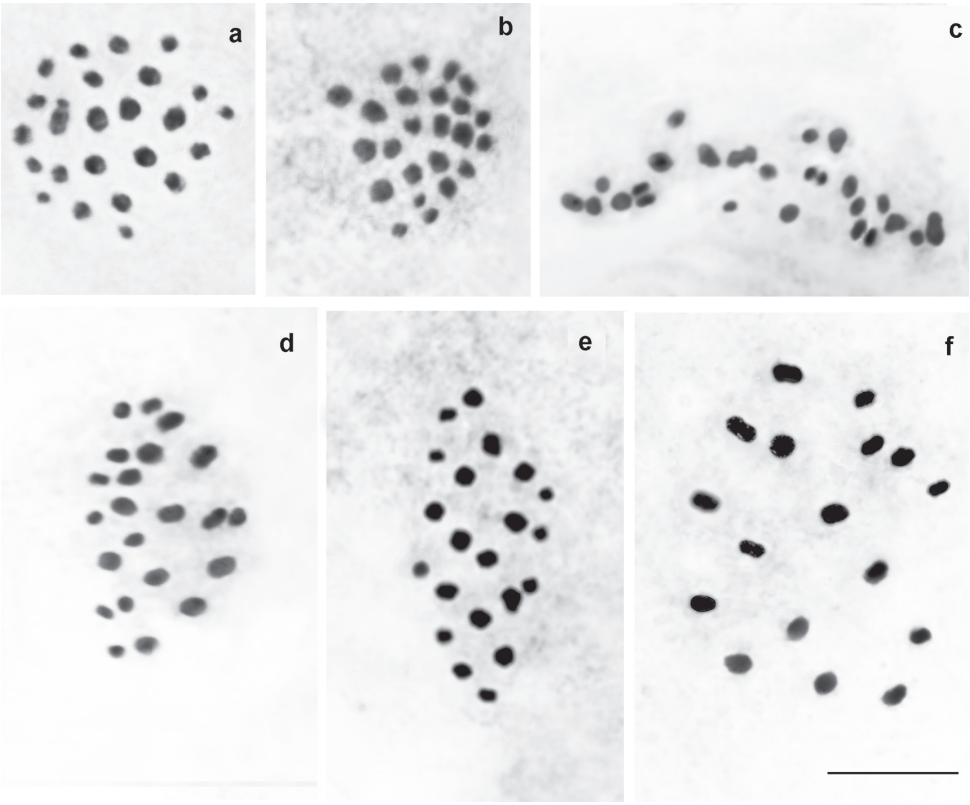

Figure 7. Karyotypes of *P. zarathustra*, *P. arasbarani*, *P. lukhtanovi* and *P. shamil.* **a** *P. zarathustra*, sample Z401, MI, n=24 **b** *P. arasbarani arasbarani*, sample N98, MI, n=24 **c** *P. arasbarani neglectus,* sample Q457, MI, n=ca25-26 **d** *P. lukhtanovi*, sample F875, MI, n=22 **e** *P. lukhtanovi*, sample H717, MI, n=21 **f** *P. shamil*, sample F958, MI, n=17. Bar = 10 μ.

similar elements were found not only at the MI (Fig. 6a, b), but also at the MII stage (Fig. 6c), most likely due to the second way of the first meiotic division.

The fact that the discovered fusions/fissions can exist in populations in both homo- and heterozygous conditions indicates, most likely, that these rearrangements can pass through meiosis and are not strongly underdominant. Previously, the low or no underdominance of chromosomal fusions/fissions was demonstrated for butterflies of the genus *Leptidea* Billberg, 1820 (Lukhtanov et al. 2011, Šíchová et al. 2015, 2016). In the *Agrodiaetus* subgenus the low underdominance of chromosomal fusions/fissions was indirectly demonstrated through analysis of homoploid hybrid speciation in *P. karindus-P. morgani-P. peilei* species complex (Lukhtanov et al. 2015b). Particularly, the formation of the diploid hybrid species *P. peilei* Bethune-Baker, 1921 had to include a hybrid ancestor heterozygous for at least 41 single chromosome fusions/fis-

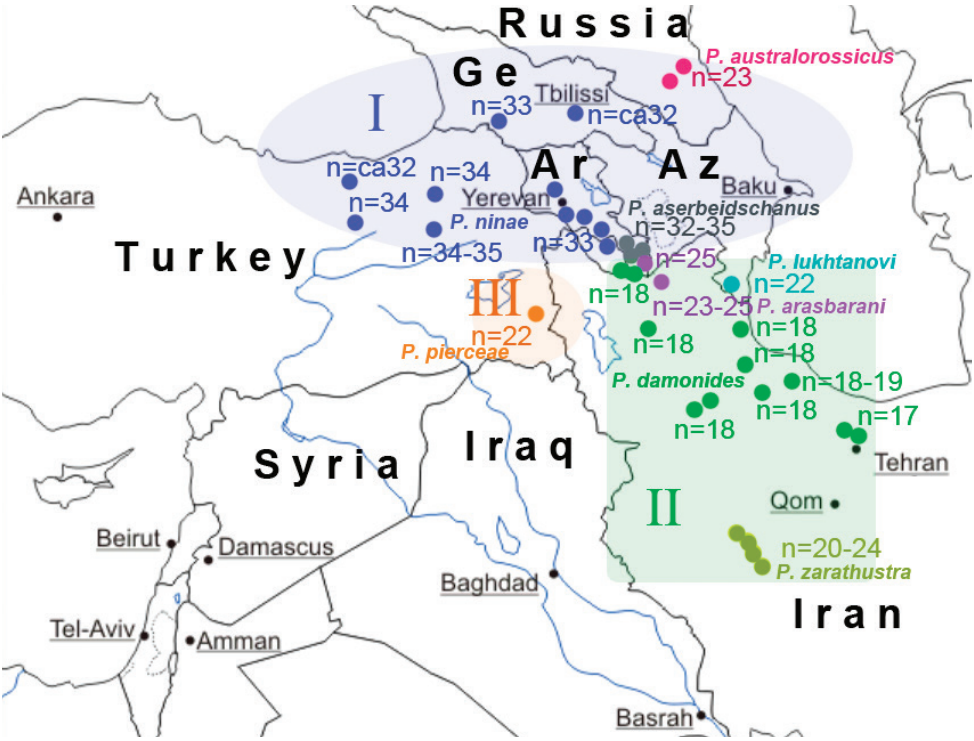

Figure 8. Distribution of *COI* haplogroups (I – III) and haploid chromosome numbers (n) in the *P. damonides* species complex.

sions, and this ancestor was at least partially fertile (Lukhtanov et al. 2015b). The low underdominance of the chromosomal fusions/fissions does not mean that these rearrangements are unimportant for the formation of reproductive isolation and speciation. The accumulation of multiple fusions/fissions can reduce gene flow between chromosomally divergent populations not only via (i) the hybrid-sterility mechanism (when chromosomal rearrangements reduce fertility of chromosomal heterozygotes), but also via (ii) the suppressed-recombination mechanism (even if chromosomal rearrangements are neutral and do not influence fertility of chromosomal heterozygotes) (Faria and Navarro 2010). Comparative phylogenetic analyses demonstrates that the second mechanism is more probable in *Agrodiaetus* (Vershinina and Lukhtanov 2017), and the gradual accumulation of chromosomal fusions-fissions can certainly drive speciation (Lukhtanov et al. 2005, Kandul et al. 2007).

Thus, the fixed differences in karyotype are not only (syn)apomorphic characters demonstrating that chromosomal races represents distinct phylogenetic lineages, i.e. species from the point of view of phylogenetic species concepts, but also indirect evidence for at least partial reproductive isolation.

COI differentiation and taxonomy of the *P. damonides* complex

The studied complex demonstrates a high level of chromosomal differentiation between taxa and a relatively low level of differentiation with respect to the mitochondrial *COI* gene, with many distinct taxa intermixed on the *COI* tree obtained (Fig. 1). This result is quite expected taking into account the previous studies (Wiemers 2003, Kandul et al. 2004, Wiemers and Fiedler 2007, Lukhtanov and Shapoval 2017) that demonstrated low interspecific differenciation and even the presence of shared *COI* barcodes between several distinct species of *Agrodiaetus*. For chromosomally divergent species, such a situation can be explained by (i) a high rate of diversification in *Agrodiaetus* resulting in numerous young species sharing ancestral polymorphism for *COI* and/or (ii) occasional interspecific hybridization resulting in mitochondrial introgression (Kandul et al. 2004, Lukhtanov et al. 2005, Vishnevskaya et al. 2016). For example, both explanations can be applied to explain the molecular relationship between chromosomally divergent *P. ninae* and *P. australorossicus*, although the second explanation (mitochondrial introgression) seems to be much less probable given the current geographic isolation between them (Fig. 8).

In case of the pair *P. ninae* – *P. aserbeidschanus* which are indistinguishable in both molecular and chromosomal characters, we can also hypothesize that these two nominal taxa are conspecific. These two taxa have been long time considered as distinct species because of a wrong assumption about their karyotypic differentiation (Lukhtanov 1989). However, the analysis of karyotype of *P. ninae* from its type-locality (Armenia: vicinity of Azizbekov, now Vaik) (Lukhtanov 1989) and of *P. aserbeidschanus* from its type-locality (Armenia: vicinity of Kadzharan, now Kajaran) (this study) did not reveal any differences between them, and the molecular analysis demonstrated the identity of their *COI* barcodes (although nuclear genes have not been studied yet).

However, *P. ninae* and *P. aserbeidschanus* are not identical with respect to their morphology and ecological preferences. Male specimens of *P. aserbeidschanus* (mostly collected around the type locality in South Zangezur Range) have specific dark brown coloration on the wing underside, blue ground color with violet tint on the wing upperside and significantly smaller size compared with the males of *P. ninae*. *Polyommatus aserbeidschanus* is known only from the subalpine belt of the South Zangezur mountain area and connected trophically with the *Astragalus* species preliminary determined as *Astragalus prilipkoanus* (sectio *Incani*) (Fabaceae) (Dantchenko 2010). As it was shown previously, *Astragalus* species of the sectio *Incani* are also host plants for other taxa of the *P.* (*A.*) *damonides* species group (Dantchenko 2010). Typical males of *P. ninae* are larger in size and have blue (not violet) coloration of the upper surface of the wings. Typical *P. ninae* inhabits tragacanth communities in the Vayots Dzor mountain range and its hostplant is *Astragalus montis-aquilis* (sectio Incani) (Dantchenko 2010). Despite this morphological and ecological differentiation, *P. ninae* and *P. aserbeidschanus* can be theoretically interpreted as local intraspecific forms of the same species, and further studies are required to clarify this situation.

A similar case is found in the pair *P. zarathustra* – *P. arasbarani*. These two taxa are allopatric, and similar with respect to morphology, karyotypes and *COI* barcodes.

However, they are differentiated with respect to ecological preferences: *P. zarathustra* is associated with dry areas in central Iran, whereas *P. arasbarani* is associated with meadow-like biotopes in subalpine zone of the north-west Iran. *Polyommatus arasbarani neglectus* is known only from low and middle altitude on southern slopes of the Meghri mountain range, it inhibits dry glades and clearance in an oak forest belt and trophically connected with astragalus species preliminary determined as *Astragalus fedorovi* (sectio *Incani*) (Fabaceae). This ecological differentiation does not allow synonymaizing these taxa, and further studies are required to clarify this situation, too.

Lycaena Damone var. *Damonides* Staudinger, 1899 is the oldest taxon described within the studied complex. Therefore analysis of its identity is of great importance for solving nomenclatural problems within the group. The taxon was described as a form of *Polyommatus damone* (hypothesis 1) and later considered as an entity close to *P. poseidon* (Forster 1961) (hypothesis 2), to *P. ninae* (Hesselbarth et al. 1995, p. 735, Eckweiler and Bozano 2016) (hypothesis 3) or to *P. elbursicus* (Lukhtanov in Hesselbarth et al. 1995, p. 735) (hypothesis 4) (see also Olivier et al. 1999, p. 16). Here we have analyzed the karyotype and *COI* barcodes of the samples from the type-locality (Ordubad in Nakhchivan, Azerbaijan) as well as the samples from the neighboring territory of Armenia (Meghri). Based on this material, we demonstrate that the hypothesis 4 is true. Thus, *P. damonides* appears as a taxon close and most likely conspecific with the taxon previously known as *P. elbursicus*. Therefore, we propose a taxonomic rearrangement of this group and suggest the following new combinations: *P. damonides elbursicus* Forster, 1956, comb. n. and *P. damonides gilanensis* Eckweiler, 2002, comb. n.

According to our observations *P. damonides damonides* inhabits tragacanth and *Paliurus* plant communities from 1000m. alt. (in Armenia) to 2100 m. alt. (in Nakhchivan, vicinity of Ordubad) and is trophically connected with *Astragalus ordubadensis* (sectio *Incani*) (Fabaceae) which is endemic of South Zangezur mountain range. It is also important to note that in Meghri-Ordubad region we have found sympatry/syntopy for the species pairs *P. arasbarani neglectus/P.damonides damonides*, *P. arasbarani neglectus/P. aserbeidschanus* and *P. damonides/ P. aserbeidschanus.*

New species description

***Polommatus* (*Agrodiaetus*) *australorossicus* sp. n.**
http://zoobank.org/12D80F81-ECEB-4888-B148-A0D6AD3B8BC1

Holotype (Fig. 9a, b), male, BOLD process ID BPAL2013-13, field # CCDB-17947_B06, GenBank accession number MG243366; karyotype preparation DK-27-97, n=23; Russia, Caucasus, Daghestan, Gimrinsky Range, Gunib, 42.406274°N, 46.931548°E, 1680 m, 14 August 1997, A. Dantchenko leg., deposited in the Zoological Institute of the Russian Academy of Science (St. Petersburg).

***COI* barcode sequence of the holotype** (BOLD process ID BPAL2013-13; GenBank accession number MG243366).

ACATTATATTTTATTTTTGGAATTTGAGCAGGAATAGTAG-
GAACATCCNTAAGAATTTTAATTCGTATAGAATTGAGAACTCCTGGATC-
CTTAATTGGAGATGATCAAATTTATAACACTATTGTTACAGCTCATG-
CATTTATTATAATTTTTTTTATAGTTATACCTATTATAATCGGAGGATTTG-
GTAACTGATTAGTTCCTTTAATATTAGGGGCACCTGATATAGCCTTTC-
CACGACTAAATAATATAAGATTCTGATTATTACCGCCATCATTAATAC-
TACTAATTTCCAGAAGAATTGTAGAAAATGGAGCAGGAACAGGATGAA-
CAGTTTACCCCCCACTTTCATCTAATATTGCACATAGAGGATCATCTG-
TAGATTTAGCAATTTTCTCTCTTCATTTAGCAGGAATTTCTTCAATTT-
TAGGAGCAATTAATTTTATTACAACTATTATTAATATACGGGTAAATAATT-
TATCTTTTGATCAAATATCATTATTTATTTGAGCAGTGGGAATTACAG-
CATTATTATTACTTTTATCTTTACCTGTATTAGCTGGAGCAATTACCA-
TATTATTAACTGATCGAAATCTTAACACCTCATTCTTTGATCCAGCTG-
GTGGAGGAGATCCAATTTTATATCAACATTTA

Paratypes. 9 males. (1) BOLD process ID BPAL2011-13, field # CCDB-17947_B04; karyotype preparation DK-34-1-97; Russia, Caucasus, Daghestan, Gimrinsky Range, Gunib, 1800 m, 15 August 1997, A. Dantchenko leg. (2) BOLD process ID BPAL2012-13, field # CCDB-17947_B05; karyotype preparation DK-34-2-97, n=ca23; Russia, Caucasus, Daghestan, Gimrinsky Range, Gunib, 1800 m, 15 August 1997, A. Dantchenko leg. (3) BOLD process ID BPAL2014-13, field # CCDB-17947_B07; karyotype preparation DK-7-97, n=ca22; Russia, Caucasus, Daghestan, Gimrinsky Range, Gunib, 1800 m, 12 August 1997, A. Dantchenko leg. (4) karyotype preparation DK-23-97, n=23, 2n=46; Russia, Caucasus, Daghestan, Gimrinsky Range, Gunib, 1800 m, 15 August 1997, A. Dantchenko leg. (5) karyotype preparation DK-30-97, n=23; Russia, Caucasus, Daghestan, Gimrinsky Range, Gunib, 1800 m, 15 August 1997, A. Dantchenko leg. (6) karyotype preparation DK-23-97-3, n=23; Russia, Caucasus, Daghestan, Gimrinsky Range, Gunib, 1800 m, 14 August 1997, A. Dantchenko leg. (7) karyotype preparation DK-23-97-4, 2n=ca46; Russia, Caucasus, Daghestan, Gimrinsky Range, Gunib, 1800 m, 14 August 1997, A. Dantchenko leg. (8) karyotype preparation DK-27-97-2, n=23; Russia, Caucasus, Daghestan, Gimrinsky Range, Gunib, 1800 m, 14 August 1997, A. Dantchenko leg. (9) karyotype preparation n=?24; Russia, Caucasus, Daghestan, Chonkatau, V. Tikhonov leg. All paratypes are deposited in the Zoological Institute of the Russian Academy of Science (St. Petersburg).

Additional samples (no DNA, no karyotype). 10 males: Russia, Caucasus, Daghestan, Gimrinsky Range, Gunib, 1450–1950 m, 11–16 August 1997, A. Dantchenko leg.

Males. Forewing length 16.5–18.5 mm.

Upperside: Ground colour bright glossy violet blue with narrow black marginal line, marginal part of forewings and hindwings slightly dusted with black scales, discal strokes absent, veins darkened distally, costal area of the forewings white, basal part of fringe dark grey on forewings, light grey on hindwings, distal part white.

Underside: Forewing ground colour grey, submarginal row blurred, but clear visible; discoidal strokes black, bordered with white; postdiscal rows of black spots bor-

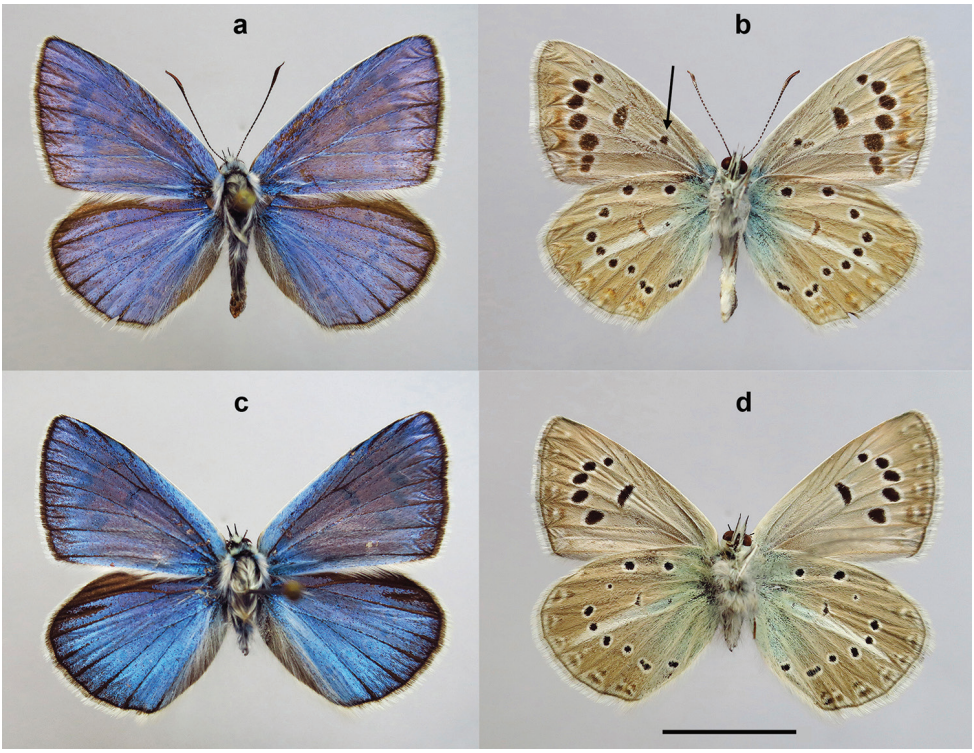

Figure 9. Specimens of *Polyommatus* (*Agrodiaetus*) *australorossicus* sp. n. and *P.* (*A.*) *shamil*. Both samples collected in Gunib (Russia, Caucasus, Daghestan, Gimrinsky Range, 1600-1800 m), 14 August 1997, by A. Dantchenko. **a, b** upperside (**a**) and underside (**b**) of the holotype of *Polyommatus* (*Agrodiaetus*) *australorossicus* sp. n. DK-27-97, n=23; arrow indicates basal black spot **c, d** upperside (**c**) and underside (**b**) of the paratype of *Polyommatus* (*Agrodiaetus*) *shamil*, CCDB-17947_B11, DK-97-18, n=17, 2n=34. Bar = 10 mm.

dered with white, 80% males have basal black spots; hindwing ground colour grey with ocherous tint, basal area with strong greenish suffusion; discal stroke less prominent than on forewings; postdiscal row of black spots bordered with white, submarginal and antemarginal marking not strong but clear visible; submarginal row bordered distally with reddish brackets, more pronounced to anal end of row; white streak sharp, equal in width; basal half of fringes pale grayish on fore- and hindwings, distal part white.

Females remains unknown.

Genitalia. The male genitalia have a structure typical for other species of the subgenus *Agrodiaetus* (Coutsis 1986).

Habitat and biology. Stony steppe and dry meadows from 1500 up to 2000 m a.s.l. Flight period: mid-July to end of August, in a single generation. The new species flights syntopically and synchronously with *P. shamil* but on average about one decade earlier. Host plant is preliminary determined as *Astragalus buschiorum* (Fabaceae). Hibernation as first instar larvae.

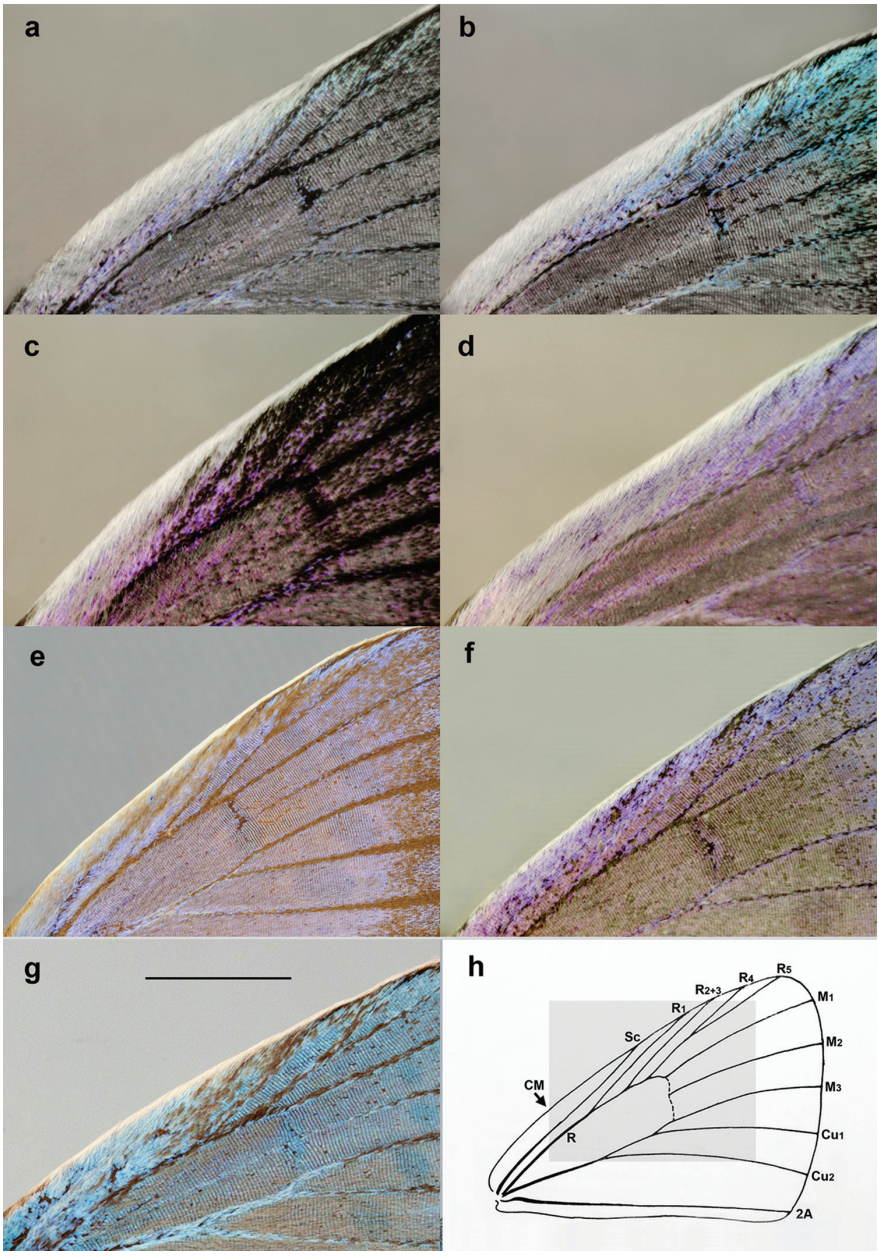

Figure 10. Pubescence of the anterior part of the forewing upper surface. **a, b, c, d** the anterior part of the forewing upper surface possesses a strong white pubescence in the area bordered by the costal margin (CM) and the veins R and R_{2+3}, i.e. this area is densely covered with relatively long white hairs (**a** *P. aserbeidschanus* **b** *P. ninae*, **c** *P. arasbarani neglectus* **d** *P. australorossicus*) **e, f** the white pubescence of the anterior part of the forewing upper surface is strongly reduced and limited to the only costal margin, the white hairs are short (**e** *P. ciscaucasicus* **f** *P. shamil*) **g** the white pubescence of the anterior part of the forewing upper surface is reduced, not dense (*P. damonides*) **h** schematic picture showing the venation of the forewing in *Polyommatus* and the photographed area (shaded). Bar = 3 mm.

Diagnosis. Phenotypically *P. (A.) australorossicus* sp. n. is practically indistinguishable from allopatric closely related *P. ninae*, *P. aserbeidschanus* and *P. lukhtanovi* but the ground colour of the underside of the hindwings is grey in the new species, with ocherous tint, not light or dark brown. The new species differs from sympatric (syntopic and synchronous) *P. shamil* (Fig. 9c, d) by specific structure of costal area of the forewings in males (Fig. 10). The submarginal row of spots on the forewing underside is more blurred (Fig. 9b), not sharp and clear visible as in *P. shamil* (Fig. 9d). Additionally, basal black spots are usually present on the underside of the forewings in *P. (A.) australorossicus* (Fig. 9b); however, this character is not constant.

Genetically *P. australorossicus* and *P. shamil* are not close. They belong to two different species groups within the subgenus *Agrodiaetus*: to *P. carmon* group (*P. australorossicus*) and to *P. cyaneus* group (*P. shamil*).

The new species differs drastically from the genetically most closely related *P. ninae* and *P. aserbeidschanus* by its karyotype (by at least 9 fixed chromosomal fusions/fissions).

The new species is similar (but not identical) to *P. lukhtanovi* (n=21-22) and *P. pierceae* (n=22) with respect to the chromosome number. However, it differs from these species by *COI* barcodes and represents a different lineage of evolution within the *P. damonides* complex.

Etymology. The name *australorossicus* is an adjective of the masculine gender. This species name originates from the Latin words "australis" (south) and "rossicus" (Russian).

Taxonomic conclusion

We propose the following taxonomic arrangement of the *P. damonides* species complex (chromosome numbers are in parentheses):

i. *Polyommatus* (*Agrodiaetus*) *ninae* lineage

P. (*A.*) *ninae* (Forster, 1956) (*Agrodiaetus transcaspica ninae* Forster, 1956; =*Agrodiaetus ninae firuze* Carbonell, 1993) (n=33-35)

P. (*A.*) *aserbeidschanus* (Forster, 1956) (*Agrodiaetus transcaspica aserbeidschana* Forster, 1956) (n=32-37)

P. (*A.*) *australorossicus*, sp. n. (n=23)

ii. *Polyommatus* (*Agrodiaetus*) *damonides* lineage

P. (*A.*). *damonides* (Staudinger,1899)

P. (*A.*) *damonides damonides* (Staudinger, 1899) (*Lycaena Damone* var. *Damonides* Staudinger, 1899) (n=18)

P. (*A.*) *damonides elbursicus* (Forster, 1956), comb. n. (*Agrodiaetus transcaspica elbursica* Forster, 1956) (n=17)

P. (*A.*) *damonides gilanensis* Eckweiler, 2002, comb. n. (*Polyommatus* (*Agrodiaetus*) *elbursicus gilanensis* Eckweiler, 2002) (n=18-19)

P. (*A.*) *lukhtanovi* (Dantchenko, 2005) (*Agrodiaetus lukhtanovi* Dantchenko, 2005) (n=21-22)
P. (*A.*) *zarathustra* Eckweiler, 1997 (*Polyommatus* (*Agrodiaetus*) *zarathustra* Eckweiler, 1997) (n=20-24)
P. (*A.*) *arasbarani* (Carbonel & Naderi, 2000)
P. (*A.*) *arasbarani arasbarani* Carbonel & Naderi, 2000 (*Agrodiaetus arasbarani* Carbonel & Naderi, 2000) (n=24-25)
P. (*A.*) *arasbarani neglectus* Dantchenko, 2000 (*Polyommatus* (*Agrodiaetus*) *zarathustra neglectus* Dantchenko, 2000; = *Polyommatus* (*Agrodiaetus*) *arasbarani ihmal* Koçak & Kemal, 2008) (n=24-26)

iii. *Polyommatus* (*Agrodiaetus*) *pierceae* lineage

P. (*A.*) *pierceae* (Lukhtanov & Dantchenko, 2002) (*Agrodiaetus pierceae* Lukhtanov & Dantchenko, 2002) (n=22)

Comment. The name *Polyommatus* (*Agrodiaetus*) *arasbarani ihmal* was suggested by Koçak and Kemal (2008) to replace *Polyommatus* (*Agrodiaetus*) *zarathustra neglectus* Dantchenko, 2000. Koçak and Kemal (2008) assumed that *Polyommatus* (*Agrodiaetus*) *zarathustra neglectus* Dantchenko, 2000 was a junior homonym of *Polyommatus neglectus* Stradomsky & Arzanov [2000], a species close to *Polyommatus icarus* (Rottemburg, 1775) described by Stradomsky and Arzanov in the second issue of the volume 7 of Izvestiya Kharkovskogo Entomologicheskogo Obschestva (p. 19) (Stradomsky and Arzanov [2000]). This issue is dated by the year 2000; however, the real date is not clear. As is written on the page 172 the issue was signed for printing on December 21, 1999, but the day when it was really printed and became accessible is unknown. This issue appeared in the library of the Zoological Institute of the Russsian Academy of Science on July 18, 2000. Thus we assume that it was published between December 21, 1999 and July 18, 2000.

The volume 48 of Neue Entomologische Nachrichten with description of *Polyommatus* (*Agrodiaetus*) *zarathustra neglectus* Dantchenko, 2000 was published and distributed in May 2000. Additional studies are required to clarify what taxon (described by Dantchenko or described by Stradomsky and Arzanov) was published first. Until this situation is resolved in a future revision, we see no other way than to use *P.* (*A.*) *arasbarani neglectus* Dantchenko, 2000 as a valid name.

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Supplementary material 1

Table S1

Authors: Vladimir A. Lukhtanov, Alexander V. Dantchenko

Data type: PDF table.

Explanation note: Chromosome numbers of the studied *Polyommatus* (*Agrodiaetus*) samples with their IDs, localities, dates and collectors.

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RESEARCH ARTICLE

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"European" race-specific metacentrics in East Siberian common shrews (*Sorex araneus*): a description of two new chromosomal races, Irkutsk and Zima

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Abstract

Karyotype studies of common shrews in the vicinity of Lake Baikal (Irkutsk Region, Eastern Siberia) resulted in the description of two new chromosomal races of *Sorex araneus* Linnaeus, 1758 (Lypotyphla, Mammalia), additional to 5 races formerly found in Siberia. In the karyotypes of 12 specimens from 3 locations, the polymorphism of metacentric and acrocentric chromosomes of the Robertsonian type was recorded and two distinct groups of karyotypes interpreted as the chromosomal races were revealed. They are geographically distant and described under the racial names Irkutsk (Ir) and Zima (Zi). Karyotypes of both races were characterized by species-specific (the same for all 74 races known so far) metacentric autosomes *af, bc, tu* and *jl*, and the typical sex chromosome system – XX/XY_1Y_2. The race-specific arm chromosome combinations include three metacentrics and four acrocentrics in the Irkutsk race (*gk, hi, nq, m, o, p, r*) and four metacentrics and two acrocentrics in the Zima race (*gm, hi, ko, nq, p, r*). Within the races, individuals with polymorphic chromosomes were detected (*g/m, k/o, n/q, p/r*). The presence of the specific metacentric *gk* allowed us to include the Irkutsk race into the Siberian Karyotypic Group (SKG), distributed in surrounding regions. The Zima race karyotype contained two metacentrics, *gm* and *ko*, which have been never found in the Siberian part of the species range, but appear as the common feature of chromosomal races belonging to the West European Karyotypic Group (WEKG). Moreover, the metacentrics of that karyotype are almost identical to the Åkarp race (except the heterozygous pair *p/r*) locally found in the southern Sweden. One of two Siberian races described here for the first time,

the Zima race, occurs in an area considerably distant from Europe and shares the common metacentrics (*gm, hi, ko*) with races included in WEKG. This fact may support a hypothesis of independent formation of identical arm chromosome combinations due to occurrence of the same centric fusions in different parts of the species range.

Keywords
karyotype, chromosomal race, Robertsonian translocation, *Sorex araneus*, Eastern Siberia

Introduction

The common shrew *Sorex araneus* Linnaeus, 1758 (Soricidae, Lypotyphla, Mammalia) is a small insectivore mammal and one of large-sized species of the genus. The species is distributed across northern Eurasia from British Isles up to the south-eastern part of Yakutia (Eastern Siberia, Russia) (Zaitsev et al. 2014). Since the 1970s, karyotype polytypy of the species has been known and dozens chromosomal races (currently 74 races known) have been described in different parts of the species range (Shchipanov and Pavlova 2016) according with the rules of the International *Sorex araneus* Cytogenetic Committee, ISACC (Hausser et al. 1994). However, there are still "white spots" where karyotypic status of common shrews remains to be unknown, for instance, the northern and eastern parts of the species range.

Chromosomal differences in polymorphic common shrew result from centric (Rb) fusions of two acrocentric chromosomes into a bi-armed metacentric or alternatively, fissions of a metacentric into two acrocentrics. Ten acrocentric chromosomes (*g, h, i, k, m, n, o, p, q, r*) are involved in both Rb translocations and WART, and different combinations of those acrocentrics cause current karyotypic diversity in the species.

It has been found that the distribution of races with similar karyotypes does not seem to be random, most likely due to common ancestry, and usually neighbor races share one or more of the same metacentrics. Initially, only three phylogenetic groups had been described: West European, East European and Siberian (Searle 1984; Wójcik 1993; Ivanitskaya 1994). Afterwards, 49 known races have been combined into four main karyotypic groups: the West (WEKG), East (EEKG) and North (NEKG) European, and Siberian (SKG) (Searle and Wójcik 1998). The existence of a separate group of races, the NEKG, was first supposed by Fredga (1987). Later, there was an assumption about the relationship between the Scandinavian and Siberian races (Halkka et al. 1994), and this was confirmed by the findings of new chromosomal races in the Ural Region (Polyakov et al. 1997; 2000b). It is very important to note that although all 10 race-specific chromosomes were used for the cladistic analysis, the combinations including only three large arms – *g, h,* and *i* were responsible for the separation of races into individual karyotypic groups (Searle and Wójcik 1998).

It has recently been shown that two centers of high karyotypic diversity of races occur in Russia (Shchipanov and Pavlova 2017). One center is located near the border of Last Glacial Maximum glacier (near the Baltic Sea) while the other one lies near Lake Baikal in Eastern Siberia. Despite less karyotypic data being available from the

second center in comparison with the Baltic area, the level of chromosomal variation was found to be significant in both cases. Nevertheless, it is obvious that new data on karyotypic diversity from the easternmost part of the common shrew range will allow us to provide more comprehensive comparative analysis.

In this paper we present data on karyotypic variation of shrews collected from a so far unstudied area in Eastern Siberia (Russia), and discuss a hypothesis whether chromosomal translocations result in the appearing of the same arm combinations in geographically remote races independently.

Material and methods

Common shrews were collected at three localities in the south-eastern part of the Irkutsk Region, Eastern Siberia, Russia: 1) 17 km SW of the Zima city on the left bank of the River Oka; 2) 17 km E of the Irkutsk city (23th km of the Goloustnoe tract) on the left bank of the River Angara; 3) 5 km SE of the Bayanday village on the left bank of the River Angara. Capture locations were determined using a GPS (Garmin) personal navigation system. A total of 22 common shrews were trapped by home-made live-traps (Shchipanov 1986; Shchipanov et al. 2008) in July-August 2016; karyotypes were obtained from 12 individuals (Table 1).

Mitotic chromosome preparations were made in the field from the bone marrow and/or spleen after colchicine treatment *in vivo* following generally Ford and Hamerton (1956) method with some modifications. Briefly, animals were injected intraperitoneally with 0.25 ml of 0.04% colchicine solution for 1-1.5 hours, then cells were washed out in a 10-ml vial by warm phosphate-buffered saline (PBS, Paneco, Russia) using a 2-ml injection syringe; incubated with 5 ml of 0.56% KCl solution for 20 min at 37°C; and fixed with freshly prepared cold glacial acetic-methanol (1:3) for 30 min then twice for 10 min. The cells are concentrated for each change of reagent by centrifuging for 5 min at 100 g.

The trypsin–Giemsa staining technique of Král and Radjabli (1974) was used for identification of each chromosome arm by G-bands. The racial status of each individual was determined according to the standard nomenclature for the karyotype of the common shrew (Searle et al. 1991) and described in terms of the metacentrics (e.g. *pr*), free acrocentrics (*p, r*), or heterozygous state (*p/r*) in the variable autosomal arms *g* to *r*. The nucleolus organiser regions (NORs) were detected by silver nitrate staining following Graphodatsky and Radjabli (1988).

Results

All studied individuals had karyotypes typical for the common shrew (Table 1). The two large (*af, bc*), one medium (*jl*) and one small (*tu*) biarmed pairs of autosomes are invariant and the same in all known chromosomal races over the species range

Table 1. New karyotypic data on common shrews from Eastern Siberia (only race-specific chromosomes indicated). Polymorphism for Rb translocation is indicated by slash (/). 2*n*A – diploid number of autosomes.

Site	Locality name	Lat/Lon	Number of specimen, sex	2*n*A	Karyotype	Race
Irkutsk Region						
1	*Zima*	53°51'10"/ 101°49'27"	2 f, 1 m	20	*gm, hi, ko, nq, p, r*	ZIMA
			1 m	20	*gm, hi, ko, n/q, p/r*	
			1 m	21	*gm, hi, ko, n/q, p, r*	
			1 f	21	*gm, hi, k/o, nq, p, r*	
			1 m	21	*g/m, hi, ko, nq, p, r*	
			1 f	22	*g/m, hi, ko, n/q, p, r*	
2	*Irkutsk*	52°17'24"/ 104°41'54"	3 m	22	*gk, hi, nq, m, o, p, r*	IRKUTSK
3	*Bayanday*	52°59'31"/ 105°40'10"	1 f	23	*gk, hi, n/q, m, o, p, r*	IRKUTSK

(Fig. 1). The typical sex chromosome system, XX and XY_1Y_2 in females and males, respectively, was found in all karyotypes. The diploid number (2n) differs between specimens due to different sex chromosomes and possible polymorphism in the autosome complement.

Two different types of race-specific autosome sets (the variable part of a karyotype) were determined using G-banding: shrews from site 1 had the arm combination of metacentrics *gm, hi, ko, nq* and two acrocentrics *p* and *r*, whereas all other individuals (sites 2, 3) were characterized by three metacentrics *gk, hi, nq* only and four acrocentrics *m, o, p* and *r* (Table 1). Only six of 12 individuals examined had homozygous karyotypes (three in site 1 and three in site 3), while the others were Rb heterozygotes, i.e., they had one or two polymorphic metacentrics and *n/q* variant was most frequent. No Rb heterozygous karyotypes found among the shrews from the site 2.

Silver nitrate staining was applied to confirm the G-banding results of chromosome arms *m* and *o*; the localization of NORs was revealed on the chromosome arm *o* in the metacentric *ko* as well as at the terminal ends of the chromosome arms *q*, *t* and *u*.

Following the rules of the standard nomenclature for *S. araneus* karyotype proposed by Hausser et al. (1994), we give the description of two new chromosomal races of the common shrew (*Sorex araneus*):

Zima race (Zi). XX/XY_1Y_2, *af, bc, g/m, hi, jl, k/o, n/q, p/r, tu*.

Type locality. Zima city and railway station vic., Irkutsk Region, Eastern Siberia, Russia, 53°51'N, 101°49'E.
Distribution. Type locality only.

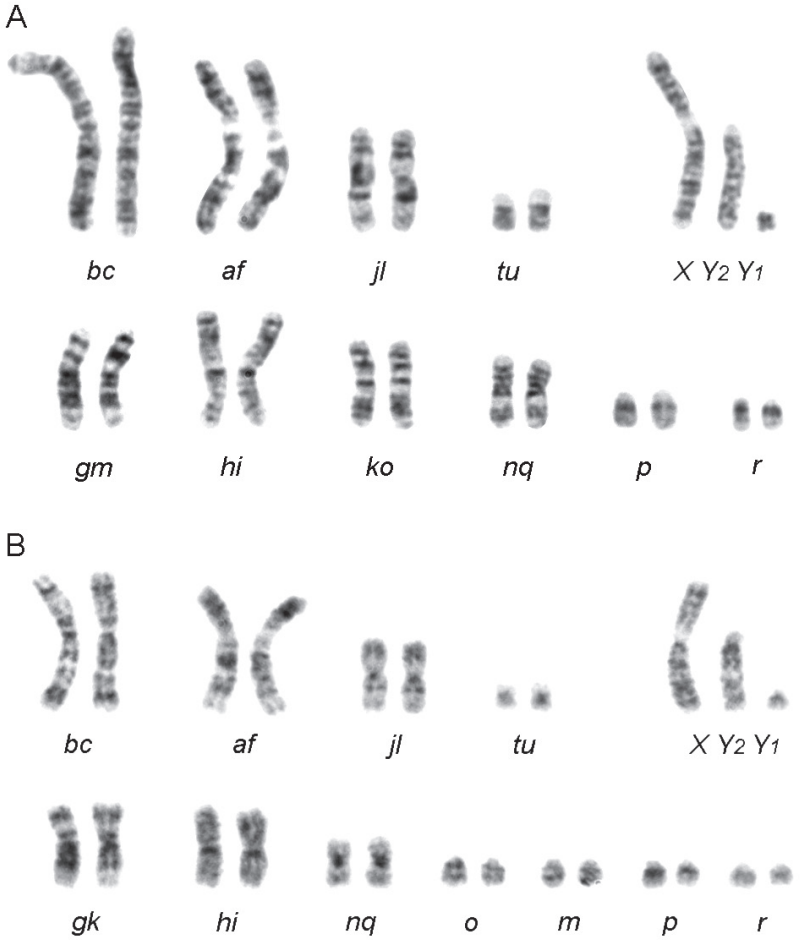

Figure 1. G-banded karyotypes of *S. araneus* males of the Zima 2n=23, FNa=20 (**A**) and Irkutsk 2n=25, FNa=22 (**B**) races. XY_2Y_1 – sex chromosomes.

Additional information. The same karyotype as the Åkarp race except chromosome arms *p* and *r* presented mostly as free acrocentrics in the Zima race (a single individual with *p/r* was found).

Irkutsk race (Ir). XX/XY_1Y_2, *af, bc, gk, hi, jl, n/q, m, o, p, r, tu*.

Type locality. Irkutsk city vic., Irkutsk Region, Eastern Siberia, Russia, 52°17'N, 104°41'E.
Distribution. Known from two sites. The range presumably is located in the south-eastern part of Irkutsk Region.

Discussion

Among all 12 analyzed shrews we determined two main karyotypic variants which differ by combination of Rb metacentrics – *gm* and *ko,* and *gk*.

Despite some polymorphic metacentrics determined among shrew karyotypes from the vicinity of Zima city (site 1) (Table 1), the arm combination of the first variant (*gm, hi, ko, nq, p/r*) is unique and the sample represents a new chromosomal race. Almost the same karyotype has been previously recorded in the Åkarp race (*gm, hi, ko, nq, pr*) distributed in southern Sweden (Fredga and Nawrin 1977). Contrary to homozygous karyotypes of the Åkarp race, almost all studied shrews from Siberia were heterozygotes (*g/m, k/o, n/q, p/r*). Also, all karyotypes, except a single individual, contained the chromosomes *p* and *r* as acrocentrics, while the Åkarp race had metacentric *pr*. Thus, in our sampling the only stable metacentric pair was *hi*. According with the rules of ISACC (Hausser et al. 1994), the range of the Åkarp race is so remote from the area where the same chromosome arm combination of race-specific metacentrics has been found that we are able to consider studied karyotypes as a new race. The race is titled "Zima" following the name of the nearest city and railway station.

The second karyotypic variant (*gk, hi, nq, m, o, p* and *r*) was determined among shrews collected from south-easternmost part of Irkutsk Region (the southwestern bank of Lake Baikal). Except a single individual from site 3 (Table 1) with polymorphic pair *n/q*, all other karyotypes were homozygous. Because this autosomal arm constitution is different from any other known *S. araneus* karyotypes, we describe this population as another new chromosomal race named after the nearest big city of Irkutsk.

Until recently, five chromosomal races (except the now invalid Altai race, Polyakov et al. 2003) have been discovered in easternmost part of species range (Eastern Siberia) – Tomsk, Ilga, Yermakovskoie, Strelka and Baikal (Polyakov et al. 2000a; Sheftel et al. 2016). Of the races, the Baikal and Strelka races have the fewest race-specific metacentrics, *hi* and *go* and *hi*, respectively. Other three races are characterized the metacentric *gk* which is a marker of the Siberian karyotypic group (SKG), thus, the Irkutsk race (*gk* and *hi*) certainly belongs to the group.

Regarding the Zima race (*gm, hi* and *ko*), the picture is more complicated because *gm* and *hi* metacentrics mark the races of the West European Karyotypic group (WEKG). The most eastern European races carrying metacentric *gm* are the Mologa and Penza races and the Kirillov race which are distributed on the right bank alongside the River Volga and the River Mezen, respectively (up to a longitude of 50°). The metacentric *hi* has been found in the race-specific karyotypes belonging to both the WEKG and Siberian group; however, it has been suggested that the metacentric could have originated independently in each of such spatially remote groups (Searle and Wójcik 1998). Thus, beyond the European part of the species range, we identified the *gm* and *ko* metacentrics in *S. araneus* karyotypes for the first time.

There are some cases when a chromosomal race inhabits an area beyond the main range of a group. For example, all neighbors of the Neroosa race (*go, hi*) distributed in European Russia belonged to another group (WEKG). The same picture can be found for the Strelka race that has the metacentric *go* but all surrounding races belong to the

SKG (except the Zima race with *gm* and *hi*). In all these cases, the isolated races are distributed close to the range of its own group, i.e. do not distant more than a range of a single race. So, we may suppose that the races within a karyotypic group have origins related to other races of a group but current isolation of ranges may be explained by hybridization or/and an impact of environmental factors.

In other cases a chromosomal race has a distribution significantly distant from the area occupied by related races of the same karyotypic group. As an example, the Bergen race inhabits the western part of Norway; while two other races having the same metacentric are distributed in Finland and the western Russia (Kalvitsa and Lemi). Similarly, the races carrying metacentric *gr* occupy an area along the southern bank of Baltic Sea, whereas recently discovered the Poyakonda race, *Py* (Pavlova 2010) inhabits the south territory of the Kola peninsula (White Sea, Russia). In both cases, it may be logical to assume the independent origin of the same metacentric variant (*gr*).

Diagnostic metacentrics *gm* and *hi* mark the WEKG, but the type locality of new Zima race is located more than 3 thousand kilometers away from the area of European races. Thus, the metacentric *gm* in the Zima race could have appeared independently during karyotypic evolution. According with the rules of the ISACC, two races having the same karyotype (the case of the Zima and Åkarp races) but isolated by distance should be considered as two different races.

There are examples when two or even three races have the same set of race-specific chromosomes and here we list some of them (Table 2; Fig. 2). It is worth to note that the first five races (cases 1 and 2) belong to the WEKG, while other four races (cases 5 and 6) – to the North European karyotypic group. It has been suggested that range expansion of a single chromosomal race in the past is more likely explanation of the current karyotypic identity of the Oxford, Sjaelland and Kirillov races than the assumption that five new metacentrics could have arose independently in karyotypes of those three races due to the same Rb fusions (Shchipanov and Pavlova 2016). In the case of the Aberdeen and Arendal races, we may assume similar scenario of the range expansion from the south of Scandinavia to the south of Britain, whereas Polyakov with co-authors (2000b) suggested that the Ilomantsi - Yuryuzan, and the Kuhmo - Sok races have independent evolutionary origin.

Here we mention the pair of the Mologa - Penza races (case 4) that have the same karyotypes. However, it should be noted that there is a probability of incorrect description of the Penza race because their karyotypes differ by the presence of polymorphism *g/m* in Penza race only and the fact of isolation of two ranges is still unclear (Orlov et al. 2007).

Also, several "acrocentric" races share identical race-specific chromosomes (cases 7 and 8), but they are occur in areas very far from each other.

The type localities of chromosomal races Åkarp and Zima (case 3) are located in very remote parts of the species range (at a distance of more than 6000 km from each other), and so it is hard to explain its current location as a result of dispersion of a single chromosomal race in the past. Moreover, in contrast to the Zima race, all studied karyotypes from Sweden were homozygous and completely metacentric. Taking into account high level of polymorphic metacentrics in the sampling from the type locality of the Zima race, it might be supposed possible ways of chromosomal evolution of studied races by accumulation of Rb translocations or WARTs. For example, a single

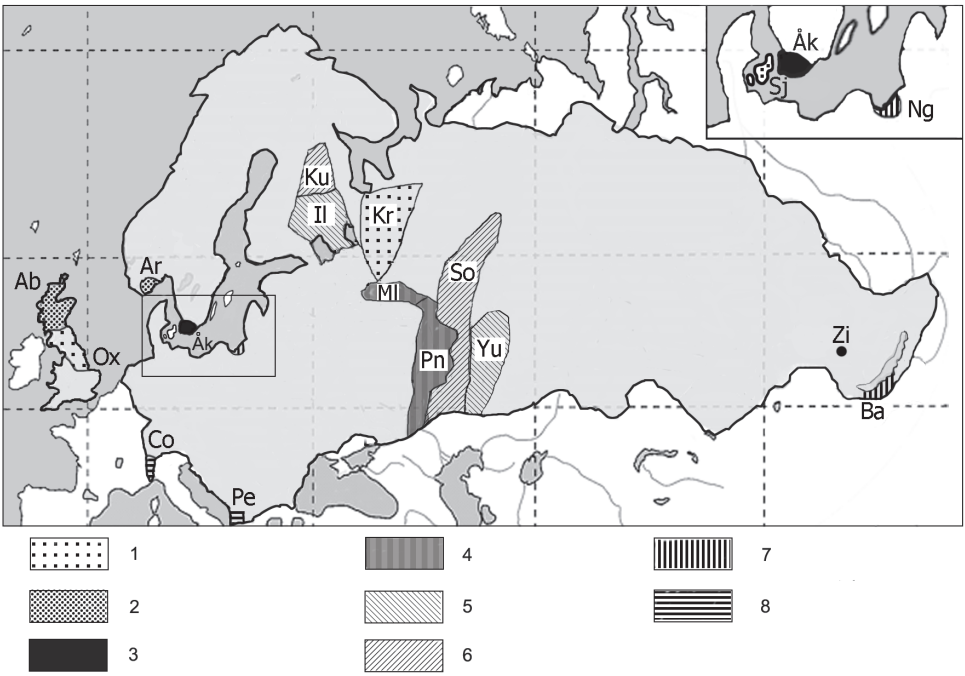

Figure 2. The distribution of chromosomal races of the common shrew sharing the same race-specific chromosomes in a karyotype (differ only by the presence of polymorphic metacentrics in some individuals): **1** Oxford (Ox) – Sjaelland (Sj) – Kirillov (Kr) **2** Aberdeen (Ab) – Arendal (Ar) **3** Åkarp (Åk) – Zima (Zi) **4** Mologa (Ml) – Penza (Pn) **5** Ilomantsi (Il) – Yuryuzan (Yu) **6** Kuhmo (Ku) – Sok (So) **7** Nogat (Ng) – Baikal (Ba) **8** Pelister (Pe) – Cordon (Co).

Table 2. Some examples of identical set of race-specific metacentrics and acrocentrics in different chromosomal races of the common shrew.

No in Fig 2	Race names	Distribution	Karyotype
1	Oxford Sjaelland Kirillov	England Denmark European Russia	*g/m, hi, k/q, no, p/r*
2	Aberdeen Arendal	England Sweden	*gm, hi, ko, np, q/r*
3	Åkarp Zima	Sweden Eastern Siberia	*g/m, hi, k/o, nq, p/r*
4	Mologa and Penza	European Russia	*g/m, hn, i/o, kr, p/q*
5	Ilomantsi Yuryuzan	N European Russia Ural mountains	*g/o, hn, i/p, k/r, m/q*
6	Kuhmo Sok	Finland European Russia	*g/o, hn, i/p, k/g, m/r*
7	Nogat Baikal	Poland Eastern Siberia	*g, hi, k, m, n, o, p, q, r*
8	Pelister Cordon	Macedonia Switzerland	*g, h, i, k, m, n, o, p, q, r*

WART is required to create the Zima race from the Strelka race or several Rb fusions in the Baikal race karyotype might also have resulted in the Zima race. Because only metacentric *hi* was found to be fixed in the studied karyotypes, we can also hypothesize that the Zima population might originate after hybridizing between the Baikal race and hypothetical full metacentric race with *gm, hi, ko, nq* and *pr*.

There is one more example of similar to the case of the Zima race. The Istranca race distributed in European Turkey has arm combination *ik*, which was not recorded in geographically close populations from south-eastern, central or western Europe but found in races distributed in north-eastern Europe and Siberia.

Thus, these examples allow us to assume the possibility of independent formation of identical chromosome arm combinations due to centric translocations in racial karyotypes of the common shrew.

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RESEARCH ARTICLE

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Characteristics of parthenogenesis in *Cacopsylla ledi* (Flor, 1861) (Hemiptera, Sternorryncha, Psylloidea): cytological and molecular approaches

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Abstract

Characteristics of parthenogenesis in *Cacopsylla ledi* (Flor, 1861) were analyzed using cytological and molecular approaches. In all three populations studied from Finland, i.e. Turku, Kustavi and Siikajoki, males were present at a low frequency but were absent from a population from Vorkuta, Russia. In a follow-up study conducted in the Turku population during 2010–2016, the initial frequency of males was ca. 10 % and showed no intraseasonal variation, but then dramatically decreased down to approximately 1–2 % level in seasons 2015–2016. Male meiosis was chiasmate with some traces of chromosomal fragmentation and subsequent fusions. In most females, metaphase in mature eggs included 39 univalent chromosomes which indicated apomictic triploidy. Only a small fraction of females was diploid with 13 chiasmate bivalents. The frequency of diploid females approximately equaled that of males. *COI* barcode analyses showed that triploid females (N = 57) and diploids (7 females and 5 males) displayed different haplotypes, demonstrating that triploid females reproduced via obligate parthenogenesis. The rarity of diploids, along with the lack of males' preference towards diploid females, suggested that most likely diploids were produced by rare triploid females which shared the same haplotype with the diploids (not found in the present analysis). Minimum haplotype diversity was detected in the Turku population, but it was much higher in Vorkuta with some indication for the mixed origin of the population. We suggest that functional diploids produced in a parthenogenetic population can give rise either to a new parthenogenetic lineage or even to a new bisexual species.

Keywords
Cacopsylla ledi, Psylloidea, apomictic parthenogenesis, triploid females, diploid females, rare males, *COI* haplotypes

Introduction

The great majority of psyllid species are characterized by bisexual reproduction. However, some members of, at least, in two genera, *Cacopsylla* Ossiannilsson, 1970 and *Trioza* Foerster, 1848 include all-female populations and are, therefore, suggested to be parthenogenetic. These are *C. ledi* Flor, 1961, *C. rara* (Tuthill, 1944), *C. myrtilli* W. Wagner, 1947, *C. myrtilli* ssp. *canadensis* Hodkinson, 1978, *T. pletschi* Tuthill, 1944 and *T. abdominalis* Flor, 1861 (Ossiannilsson 1972, Hodkinson 1976, 1978, Gegechkori 1985). Parthenogenesis of this kind is called thelytoky which is characterized by the presence of females that produce only daughters without fertilization. Thelytoky can be obligatory if only parthenogenetic populations are present throughout the whole distribution range of a particular species. Nevertheless, it can be facultative if both bisexual and parthenogenetic reproduction occurs, with cyclical parthenogenesis in aphids as a well-known example (Normark 2003, Vershinina and Kuznetsova 2016).

However, identifying facultative thelytokous taxa is usually difficult. In many animal taxa the existence of so called rare males was reported for some parthenogenetic lineages (Blackman 1972, Palmer and Norton 1990, Butlin et al. 1998, Martens 1998, Rispe et al. 1999, Simon et al. 1999, Delmotte et al. 2001, Snyder et al. 2006, Domes et al. 2007, Engelstadter et al. 2011, Maccari et al. 2013, Nokkala et al. 2013). These males can be either nonfunctional, i.e. incapable of producing haploid gametes (Taberley 1988, Nokkala et al. 2013) or functional with normal gamete production (reviewed by Maccari et al. 2013). Both kinds of males were found in geographically separate populations in the parthenogenetic psyllid *Cacopsylla myrtilli* (Nokkala et al. 2013). Females of this species are apomictic triploids (Nokkala et al. 2008). Cytological analysis has also discovered diploid females coexisting with the rare males especially at high altitude. These rare males, although being nonfunctional, copulate randomly with both parthenogenetic and "sexual" diploid females (Nokkala et al. 2015).

The characteristics of parthenogenesis in the Holarctic species *C. ledi* are poorly known. The species lives on wild rosemary *Ledum palustre* Linnaeus, 1753, and was previously recorded from Fennoscandia, the Baltic countries, Poland, Germany, Russia, Japan and Alaska (Ossiannilsson 1992). In addition to all-female populations, populations with males are known to exist, although quantitative frequencies of males are unknown. The males share the same chromosome number 2n = 25 (24 + X) with males of the genuine bisexual psyllid species (Kuznetsova et al. 1997).

In the present study, we planned to determine the frequency of males in four populations of *C. ledi*. In a follow-up study we planned to find out if the frequency of males would undergo any changes either during one reproductive season or between successive seasons. In addition, we planned to analyze chromosomes in mature eggs to

determine the type of parthenogenesis and details of reproduction types of females in a particular population. Intrapopulational relationships between the females and males in a population were analyzed by using DNA barcode sequences.

Material and methods

Samples

Specimens of *C. ledi* were collected on *Ledum palustre* in four geographically separate locations, Turku, Kustavi and Siikajoki in Finland and Vorkuta in Russia (Table 1). Since this study was focused on the Turku population, samples were collected there during 2010–2016.

Cytological study

Both female and male specimens of *C. ledi* were collected in June, July and early August to study spermatogenesis and sex ratios in certain populations. As females carried no mature eggs at that time, they were collected later in August for cytology (Table 1). Complete bodies of male individuals were put in 3:1 fixative (96 % alcohol: glacial acetic acid) or stored in 96 % alcohol in the field immediately after collection. To allow both chromosomal and haplotype analyses of the same individuals, collected living females were taken to the laboratory and dissected individually. For every female, the abdomen was put into the fixative while the head and thorax parts were stored in alcohol.

Table 1. Locations, number of females and males, male percentages and collection dates of *C. ledi* populations.

Location		Females	Males	Male percentage	Date
Kustavi	60°39'20"N, 21°18'12"E	310	4	1,3 %	1.8.2010
Siikajoki	64°39'32"N, 25°19'33"E	152	3	1,5 %	19.8.2010
Turku	60°29'56"N, 22°15'55"E	no adults			22.6.2010
		112	14	11,0 %	30.6.2010
		149	14	8,5 %	6.7.2010
		132	17	11,4 %	16.7.2010
		182	17	8,5 %	26.7.2010
		82	10	10,3 %	3.8.2010
		72	10	12,2 %	11.8.2010
		40	0	0,0 %	26.8.2011
		124	14	10,1 %	19.7.2012
		170	2	1,1 %	25.8.2015
		78	2	2,5 %	27.7.2016
Vorkuta, Russia	67°30'N, 64°02'E	10†	0	0,0 %	6.8.2013

†species confirmed by *COI* sequence among 39 individuals in the sample.

Cytology was performed following Nokkala et al. (2008). In brief, the abdomen was transferred from the fixative into a drop of 50 % acetic acid. Three to four mature eggs were extracted from the ovaries, the presence or absence of sperm in the spermatheca was recorded, and the rest of the abdomen was removed. To remove chorion, the eggs were cut in two parts with well sharpened tungsten needles. When yolk became transparent the eggs were squashed. The cover slips were removed by the dry ice method and slides were then immersed in fresh 3:1 fixative for 30 min and air dried. Air-dried slides were stained first with Schiff's reagent for 30 min and then with 5% Giemsa for 40 min. Chromosomes were photographed with a BU4-500C CCD camera (BestScope International Limited, Beijing, China) attached to Olympus BX51 microscope (Olympus, Japan) using ISCapture Software version 2.6 (Xintu Photonics Co LTD, Xintu, China). Photographs were processed with Corel Photo-Paint X5 software.

DNA barcoding

Total genomic DNA was extracted with DNeasy Blood and Tissue Kit (Qiagen) from complete bodies or thorax parts of adults. In cases when yield was below 20 ng / µl, the extractions were concentrated by precipitation with sodium acetate according to the standard procedure and the precipitate was solubilized in distilled water in one-fourth of the original elution volume. A fragment of *cytochrome c oxidase subunit I* (*COI*) gene was amplified using Applied Biosystems 2720 Thermal Cycler. Initially, the *C. myrtilli* specific primers HybCamyCO (forward) and HybymaCCO (reverse) were used and PCR reactions were carried out as described by Nokkala et al. (2015). Later on, to check the sequence at 5' and 3' ends, flanking primers HybCacoCO 5'-T7Promoter(F)-CTAACCATAARACTATTGGAAC-3' (specifically designed forward primer) and a modified HCO (Folmer et al. 1994) reverse primer HybHCOMod 5'-T3-TAAACTTCAGGGTGACAAAAAATCA-3' were used. PCR products were purified with QIAquick PCR Purfication Kit (Qiagen) and sequenced by Macrogen Europe (Amsterdam, the Netherlands). The sequences were trimmed to span a 638 bp stretch of the gene to match the available sequences of related *C. myrtilli* (Nokkala et al. 2015). Sequences obtained during this study have been deposited in GenBank under the accession numbers MF978762–MF978766.

Results

Population structure

The majority of individuals in all populations were females, while the frequency of males varied in different samples from 0 % to 12.2 % (Table 1). In Kustavi and Siikajoki, the frequencies were low, 1.3 % and 1.5 % respectively. Males were absent in the

sample from *Ledum* in Vorkuta. In the Turku population, the frequency of males was higher, around 10 %, and showed no seasonal variation between 30.6–11.8 in 2010. The frequency remained high in 2012, but decreased dramatically to 1.1 % in 2015 and remained low in 2016. Low frequencies of males suggest that *C. ledi* reproduces parthenogenetically, and males represent the so-called rare males commonly found in parthenogenetic lineages.

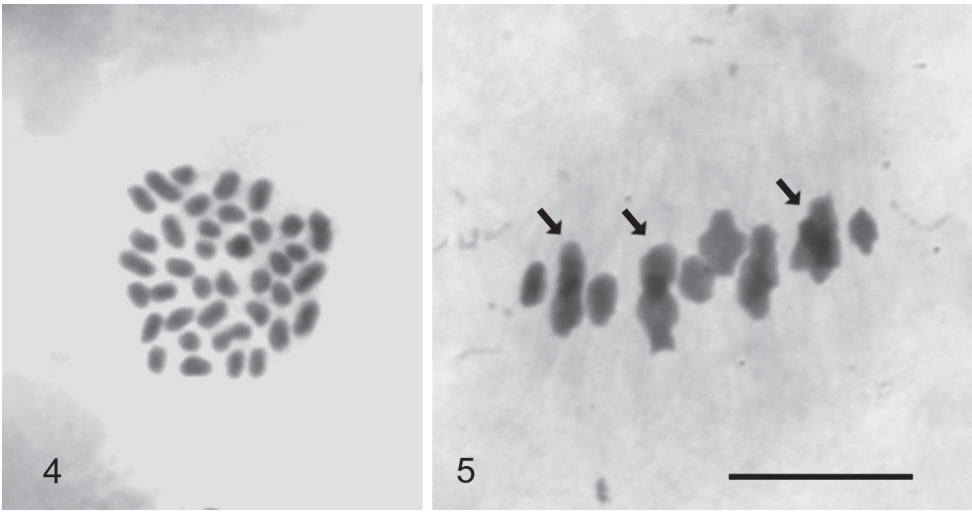
Cytology

In *C. ledi* males, testes have four testicular follicles, in contrast to two, which is the common number in psyllids (Kuznetsova et al. 2012). The male karyotype was earlier reported to be 2n = 25 (24+X) by Kuznetsova et al. (1997), which was confirmed by the present study (Fig. 1). In male meiosis chiasma formation is normal; hence twelve bivalents and a univalent X chromosome are seen at metaphase I. However, males heterozygous for fusions are not uncommon (Figs 2 and 3); they can carry either a single heterozygous trivalent (Fig. 2) or two such trivalents (Fig 3) Nevertheless, these metaphases originate from different testes of the same individual, thus indicating that the chromosome breaks leading to subsequent fusions have occurred in the germ line.

Females

The chromosome number of females is most easily determined at metaphase I in mature eggs. For a closer study of the biology of females during one season, several samples were taken from the Turku population in 2010 starting on 30.6., when adults just appeared in the population (Table 1). First females with mature eggs were found not earlier than 11.8, when single mature eggs were found in very few females (Table 2). On 25.8.2015 all females carried several mature eggs in their ovaries. The egg-lying of females was studied in the laboratory using material collected in 2015. We found that

Figures 1–3. Male meiosis in *C. ledi*. **1** Metaphase I with normal karyotype, n = 12 + X (0) (from Kuznetsova et al. 1997) **2** Metaphase I. Arrow points to trivalent which is heterozygous for a fusion **3** Metaphase I. Arrows point to two trivalents which are heterozygous for fusions. Scale bar: 10 μm.



Figures 4–5. Female meiosis in *C. ledi*. **4** Metaphase from mature egg with 39 univalent chromosomes **5** Metaphase I plate with 13 chiasmate bivalents (12 + XX), sex chromosome bivalent cannot be identified. Arrows point to three overlapping pairs of bivalents. Scale bar: 10 μm.

Table 2. Number of triploid and diploid females in Turku population.

Triploid females	Diploid females	Percentage of diploids	Collection date
2	1	N/A	11.8.2010
14	6	30,0 %	28.6.2011
36	1	2,7 %	25.8.2015
27	1	3,6 %	21.8.2016

Table 3. Number of diploid and triploid females with sperm in their spermathecae.

Number of females checked					
triploid		diploid		Σ	collection date
sperm	no sperm	sperm	no sperm		
2		1		3	11.8.2010
1	9	1	3	14	26.8.2011
1	34		1	36	25.8.2015
1	16		1	18	21.8.2016
5	59	2	5	71	totals

females preferred to deposit their eggs on the underside of the uppermost narrow leaves of *Ledum* plants. This clearly indicates that *C. ledi* hibernates as an egg.

Cytological analysis revealed that there were two kinds of females in the population. Mature eggs of the great majority of females showed 39 univalent chromosomes at metaphase, indicating that these females were apomictic triploids (Fig. 4). There

were also a small number of females with a haploid number of chiasmate bivalents, i.e. those females were diploid (Fig. 5). Typically, there was only a single terminal or subterminal chiasma per bivalent. The frequency of diploid females within a particular sample varied from 30.0 % to 2.7 %, as shown in Table 2. It is noteworthy that the approximate tenfold drop-down in the frequency of diploid females between 2012 and 2015 is similar to the decrease found in male frequencies from 10.1 % to 1,1 % (Table 1). Unfortunately, no mature eggs were found in females collected in Vorkuta.

The absence of male mating preference

With both diploid and triploid females present in a population, it is interesting to find out whether males prefer to mate with diploid ones or, at least, can distinguish between these two. For this purpose, while making cytological preparations from females, spermathecae were also checked for the presence of sperm. However, only seven out of 71 females checked carried sperm in their spermathecae, two of them being diploids and the remaining five triploids (Table 3). This indicates that males are quite inactive in mating in general but occasionally mate either with diploids or triploids. Since the males do not discriminate against triploid females successful independent bisexual reproduction in *C. ledi* seems highly unlikely.

COI barcoding

DNA was isolated from alcohol preserved thorax parts of the cytologically studied individuals. A *COI* fragment of 638 nucleotides was sequenced from 57 triploid parthenogenetic females and 12 diploids (7 females and 5 males) from the Turku population. All triploids shared the same haplotype which was different from that of the diploids. These haplotypes differed by a particular transversion at the position 192, T (Turku 1 haplotype, MF978762) in triploids and A (Turku 2 haplotype, MF978763) in diploids. These data, therefore, demonstrated that triploid parthenogenetic from Turku produced exclusively triploid offspring. The haplotype diversity in the Turku population was low, but was much higher in Vorkuta. Turku 1 haplotype was also found in Vorkuta (2/11 females). In addition, three specific haplotypes from Vorkuta were found, Vorkuta 1 (1/11 females), Vorkuta 2 (5/11 females) and Vorkuta 3 (3/11 female) differing from Turku 1 by either a single nucleotide (Vorkuta 1 and 2) or three nucleotides (Vorkuta 3) (MF978764–MF978766).

Discussion

Our findings show that details of parthenogenesis in *C. ledi* are similar to those found previously in *C. myrtilli* (Nokkala et al. 2015). Parthenogenetic females of *C. ledi* are

triploid showing apomictic oogenesis in which normal meiosis is replaced by a modified mitosis. In addition to triploid females, there were also diploids showing normal chiasmate meiosis. Moreover, the presence of diploid females among obligate triploid parthenogenetic females was discovered for the first time in populations of *C. myrtilli* collected at various altitudes on the hill Rindhovda in southern Norway (Nokkala et al. 2015). Diploid females showing conventional meiosis were found at frequencies similar to those of rare males at three different altitudes. The highest percentage of both diploid females and males, 10%, was found in the high altitude (1000 m) population, above the tree line. Comparison of frequencies of diploids at different altitudes showed that environmental factors, like altitude, seem to significantly affect the production of diploids (Nokkala et al. 2015). The findings that rare males in those populations were nonfunctional, producing only diploid sperm (Nokkala et al. 2013), on one hand, and the fact that diploids showed the same *COI* haplotype as triploid females, on the other hand, collectively suggested that diploids resulted from reversions from triploidy and that the frequency of these reversions seemed to be influenced by environmental factors, e.g. altitude. In addition, reversions resulted in both males and diploid females with a similar frequency (Nokkala et al. 2015).

Haplotype diversity in the Turku population of *C. ledi* was extremely low, where the two recorded haplotypes differed by a single transition. However, much higher diversity was found in the Vorkuta population showing four different haplotypes among the small number of females studied. The close similarity of haplotypes Vorkuta 1 and Vorkuta 2 to that of Turku 1 suggests common ancestry. In contrast, Vorkuta 3 haplotype differed from all other haplotypes found in the population by three nucleotide changes, one of these being a transversion, indicating different origin of this haplotype.

In *C. ledi* triploid females and diploid females and males displayed different *COI* haplotypes. Our results prove that triploid females reproduce via obligate thelytoky. Those females, therefore, do not produce diploid females or males. To account for the occurrence of diploid individuals, it is tempting to speculate on the possibility of independent bisexual reproduction. Potentially, reproduction of this kind is possible, since diploid females display normal chiasmate meiosis and males despite some disturbances show virtually normal meiosis. Two observations, however, make bisexual reproduction unlikely. Firstly, the frequency of males in populations at Turku after the drop-down of diploids is very low and is the same magnitude or clearly below 10% like that of rare males in oribatid mites (Palmer and Norton 1990), in *Artemia* Leach, 1819 species (Maccari et al. 2013), or in *C. myrtilli* populations (Nokkala et al 2013). Secondly, those males do not discriminate against triploid females making bisexual reproduction highly unlikely. An alternative explanation implies that rare, still undiscovered triploid females which share the same haplotype with diploids are responsible for the production of diploid females and males via reversion from triploidy to diploidy. This means that the genotypes of triploid females would greatly affect their ability to produce diploids. In turn, this would also mean that *C. ledi* is an obligate parthenogenetic. However, additional studies are needed to confirm this suggestion.

Although in the short term it is difficult to see any advantage for the production of diploids in the long run they can provide further evolutionary opportunities for a par-

thenogenetic taxon. It is known that functional rare males can mate with closely related sexual females to give rise to a new parthenogenetic lineage. This type of parthenogenesis, known as contagious parthenogenesis (Simon et al. 2003), was analyzed in detail in *Daphnia pulex* Leydig, 1860 (Paland et al. 2005) and brine shrimps of the genus *Artemia* (Maccari et al. 2013). The origin of contagious parthenogenesis was also proven in the laboratory for *D. pulex* (Innes and Herbert 1988), for the aphid *Myzus persicae* (Sulzer, 1776) (Blackman 1972) and for *Artemia* spp. (Maccari et al. 2014). Apparently, new parthenogenetic lineages of contagious origin can also be produced by diploid females found in *C. myrtilli* (Nokkala et al. 2015) and in *C. ledi* (the present study), if these females are mated with males of a coexisting sexual species. The origin of contagious parthenogenesis could more easily occur via diploid females than through males as they are functional (or show normal meiosis) even in the case when males are nonfunctional (Nokkala et al. 2015). If both diploid females and functional rare males exist in a population, mating between diploids could occasionally result in diploid offspring, in spite of the fact that males do not discriminate against triploid females. Potentially, this could lead to the origin of a new bisexual species from a parthenogenetic lineage. This can also be the case in oribatid mites. Domes et al. (2007) considered phylogenetic relationships between certain taxa belonging to seven families of the oribatid mite group Desmonomata and concluded that a parthenogenetic lineage could evolve to a new parthenogenetic lineage or even to a new bisexual species. Apparently, the evolutionary potential of parthenogenetic lineages is high, complex and far more diverse than previously understood.

Conclusion

Parthenogenetic females of *C. ledi* are triploid with apomictic meiosis. Triploid females reproduce via obligate parthenogenesis. We also suggest that rare males and diploid females, if present, are produced by triploid females by reversions from triploidy to diploidy. This probably demonstrates how a parthenogenetic taxon can give rise either to a new parthenogenetic lineage or even to a new bisexual species.

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CORRIGENDA

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After the publication of our article, we detected an error in the figure 3. On the ideogram, the signal of 5S rDNA loci illustrated with a striped signal over the long arm of chromosome 2 at proximal/interstitial position is not observed. Correct figure is as follows:

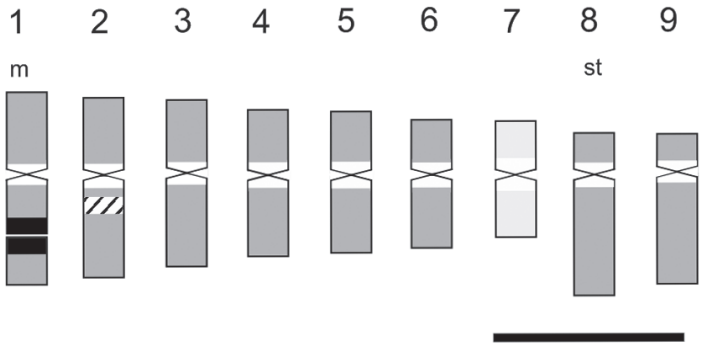

Figure 3. Ideogram of *A. porphyrocalyx* performed with measures of chromosomes obtained by classical technique. The "A" chromosome is represented with light grey colour. Distribution of 5S rDNA loci is illustrated with a striped signal and that of 18S–26S rDNA loci with a black signal. Heterochromatic regions counterstained with C-DAPI+ are represented with white bands. Scale = 2 μm.

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Chromosomal assignment of centromere-specific histone CENH3 genes in rye (*Secale cereale* L.) and their phylogeny

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Abstract

Centromeres are essential for correct chromosome segregation during cell division and are determined by the presence of centromere-specific histone 3 (CENH3). Most of the diploid plant species, in which the structure and copy number of *CENH3* genes have been determined, have this gene as a singleton; however, some cereal species in the tribe Triticeae have been found to have CENH3 in two variants. In this work, using the set of the wheat-rye addition lines we wanted to establish the chromosomal assignment of the *CENH3* genes in the cultivated rye, *Secale cereale* (Linnaeus, 1753), in order to expand our knowledge about synteny conservation in the most important cereal species and about their chromosome evolution. To this end, we have also analyzed data in available genome sequencing databases. As a result, the αCENH3 and βCENH3 forms have been assigned to rye chromosomes 1R and 6R: specifically, the commonest variants αCENH3-v1 and βCENH3-v1 to chromosome 1R, and the rare variants, αCENH3-v2 and probably βCENH3-v2, to chromosome 6R. No other CENH3 variants have been found by analysis of the rye genome sequencing databases. Our chromosomal assignment of CENH3 in rye has been found to be the same as that in barley, suggesting that both main forms of CENH3 appeared in a Triticeae species before the barley and wheat-rye lineages split.

Keywords

Centromeric histone CENH3, rye, wheat-rye addition lines, barley, Triticeae

* these authors contributed equally to the paper

Introduction

In centromeric nucleosomes, canonical histone H3 appears in the form of its centromere-specific modification denoted in plants as CENH3. The presence of this protein is by far the most distinct molecular feature of this chromosomal region. Unlike canonical histone H3, which has a conserved structure, CENH3 normally shows considerable variability across species (Sanei et al. 2011, Maheshwari et al. 2015, Neumann et al. 2015). Different domains of this molecule are diverging differently. An extended N-terminal tail (NTT) domain and loop 1 of the histone fold domain (HFD) putatively interact with centromeric DNA (Vermaak et al. 2002) and show signatures of positive selection in some animal and plant species (Malik and Henikoff 2001, Talbert et al. 2002), while the part of the HFD that lies outside loop 1 is generally conserved (Talbert et al. 2002, Cooper and Henikoff 2004, Hirsch et al. 2009, Finseth et al. 2015).

Most of the diploid plant species, in which the structure and copy number of *CENH3* have been determined, have this gene as a singleton. Cereal species as these are, for example, maize and rice (Zhong et al. 2002, Nagaki et al. 2004), which are phylogenetically distant from the tribe Triticeae (the subfamily Pooideae, Dumort, 1824), which includes the closely related genera *Triticum* Linnaeus, 1753, *Secale* Linnaeus, 1753 and *Hordeum* Linnaeus, 1753. The closest relatives to rye in Triticeae, namely, tetraploid wheat species (Yuan et al. 2015), diploid barley, wheat and *Aegilops* Linnaeus, 1753 species (Sanei et al. 2011, Yuan et al. 2015), have been found to have *CENH3* in two variants. Thus, the presence of at least two copies of the *CENH3* gene is a shared feature of the species in the tribe Triticeae, and this gene had probably been duplicated before the barley and wheat-rye lineages split, which is variously reported to date back to 8-9 MYA (Middleton et al. 2014) or 11.6 MYA (Martis et al. 2012). Using wheat-barley addition and substitution lines, the chromosomal assignment of the *CENH3* genes in two barley species, *H. vulgare* Linnaeus, 1753 and *H. bulbosum* Linnaeus, 1756, was established as encoding by chromosomes 1H and 6H (Sanei et al. 2011). In hexaploid wheat, *CENH3* genes were assigned to chromosome 1 in all the homeologous genomes, AA, BB and DD (Li et al. 2013, Yuan et al. 2015). However, so far the chromosomal localization of the *CENH3* genes in the other Triticeae species has been beyond the scope of any study known to us.

It has been established by comparative RFLP (restriction fragment length polymorphism) that the rye genome shares extensive synteny with the barley and wheat genomes (Devos et al. 1993). Accumulation of rye genome sequencing data (Martis et al. 2013, Bauer et al. 2017) enabled a genome-wide high-density comparative analysis at a new level and identified six major translocations that had shaped the modern rye genome and made it different from a putative Triticeae ancestral genome (Martis et al. 2013). In this work, we characterize the molecular structure of the main forms of CENH3 protein in rye, *Secale cereale*. Also, we attempt to assign the *CENH3* genes to particular chromosomes, using a set of seven wheat-rye addition lines, each containing a rye chromosome in the wheat genome. A comparison of the localization sites of so functionally important genes in closely related genera is expected to improve our knowledge about conservation

of synteny between the most important cereal species. To this end, available databases with the DNA sequences of rye, *S. cereale*, and diploid species as donors of the hexaploid genome of *T. aestivum* Linnaeus, 1753 have been analyzed and the phylogenetic relationships between the different variants of *CENH3* have been inferred.

Material and methods

Plant material and plant growth

The plant material used were the bread wheat 'Chinese Spring' (CS) (2n=6x=42, AABBDD), the rye cultivar Imperial (2n=2x=14, RR) and wheat-rye ('Chinese Spring'/'Imperial') disomic addition lines involving rye chromosomes 1R–7R (Driscoll and Sears 1971). 'Chinese Spring' is an international standard for wheat research, much as 'Imperial' is for rye. Seeds were from the germplasm collection of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. All plants were grown in a greenhouse at 22 °C/18 °C (day/night) with a 16-h day length.

RNA isolation and PCR amplification

Total RNA was isolated from leaves of 12-day-old seedlings using the TRI Reagent (MRC, Inc., USA) and treated by RQ-RNase-Free DNase (Promega, Madison, WI) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA using a RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The specific primers used to amplify the *CENH3* gene from cDNA were:

1) 5'-ATGGCCCGCACCAAGCAC-3', 5'-GCATCACCAAAGCCTCC-3', to amplify the coding region of αCENH3; and
2) 5'-TGGGTCGCACGAAGCAC-3', 5'-TCACCAAAGCCTTCTCCCC-3', to amplify the coding region of βCENH3.

Cloning and sequencing

RT-PCR products were purified using a Qiagen Purification Kit (Qiagen) and cloned using an InsTAclone PCR Cloning Kit (Thermo Fisher Scientific). Both strands of each of 15–20 clones of each parental variety and addition line were sequenced using an ABI 3130×1 Genetic Analyzer (Applied Biosystems Inc., CA) and an ABI BigDye Kit according to a standard protocol. Similarity searches between the *CENH3* sequences and their orthologs in other species were carried out using the TBLASTN software (Altschul et al. 1990) in the NCBI database (http://blast.ncbi.nlm.nih.gov/Database/).

Sequence alignments and phylogenetic analysis

Amino acids alignments were performed online using Clustal Omega (Sievers et al. 2011) (http://www.ebi.ac.uk/Tools/msa/clustalo) and used for downstream analysis and visualization (Waterhouse et al. 2009) (http://www.jalview.org). The phylogenetic tree based on amino acid sequences was constructed using the Neighbor Joining algorithm in MEGA6 (Tamura et al. 2013). Bootstrap values were calculated from 1000 replicates.

Analysis of databases

The search for rye genomic *CENH3* sequences was performed in among entries in the Sequence Read Archive (European Bioinformatics Institute, accession ID ERP001745) for sorted rye chromosomes 1R-7R (Martis et al. 2013). The sequences of *CENH3* in the putative A-genome donor *Triticum urartu* Thumanjan ex Gandilyan, 1972 (accessions KM507181 and KM507184), the putative B-genome donor *Aegilops speltoides* Tausch, 1837 (accessions KM507182 and KM507185), and the putative D-genome donor *Aegilops tauschii* Cosson, 1849 (accessions KM507183 and KM507186) were downloaded from NCBI. Genomic DNA sequences were aligned with *CENH3*-coding sequences by the Martinez-NW method using Lasergene's MegAlign application.

Results

Assignment of the *CENH3* variants to rye chromosomes

We characterize two main forms of CENH3 proteins, αCENH3 and βCENH3, and their variants, according to differences in size and amino acid substitutions. The α*CENH3-v1* cDNA sequence in the cultivated rye *S. cereale* is 501 bp in length and the associated protein consists of 166 amino acids. In *S. cereale*, β*CENH3-v1* is distinct from α*CENH3-v1* in that the former has several deletions in the NTT and the insertion of three nucleotides, ACC, which encode the amino acid threonine, in the HFD. Thus, β*CENH3-v1* has an overall length of 456 bp and encodes a protein made up by 151 amino acids. Most of the NTT amino acid sequences in αCENH3 and βCENH3 do not align well with each other, the alpha and beta forms share as low as 58% nucleotide identity of the first 60 nucleotides from the 5'-end. In addition to these main forms, their much less common variants (minor, throughout) were found. The α*CENH3-v2* sequences were 492 bp in length each, that is, they were shorter α*CENH3-v1*. Additionally, these two αCENH3 variants have different amino acids at some positions. Some rye accessions carry *CENH3* sequences that are individually 6 bp longer than β*CENH3-v1* and encode two additional amino acid residues of threonine in the NTT domain (Fig. 1B). We classify sequences as these as βCENH3-v2. The two βCENH3 variants have different amino acids at some positions, too.

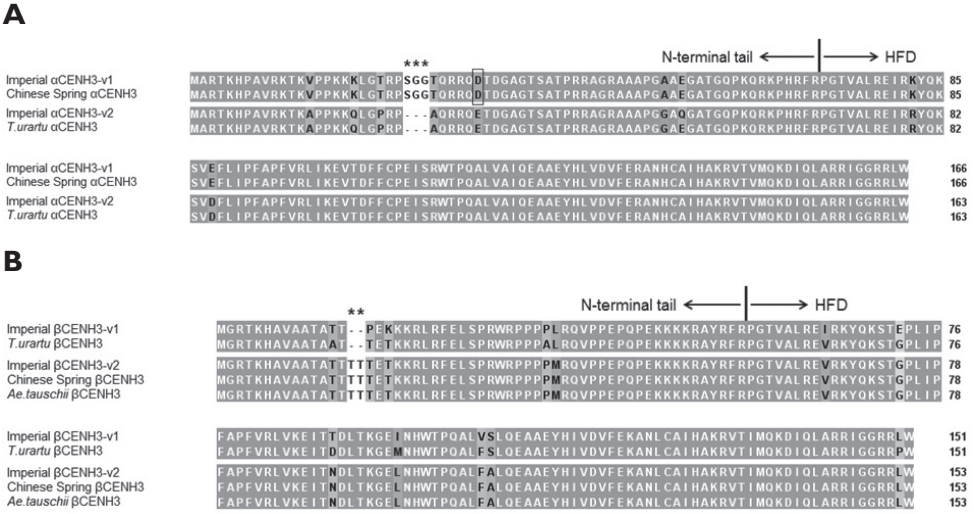

Figure 1. Examples of amino acid alignments of the different variants of the main forms of *CENH3* in rye *S. cereale* (cv. Imperial) and Triticeae species: *T. urartu* (KM507181, KM507184) and *Ae. tauschii* (KM507186). **A** αCENH3 **B** βCENH3. Asterisks are above positions with short insertions/deletions in the N-terminal tail; the position with the highest percentage of amino acid substitutions is framed.

The amino acid differences between CENH3 in rye and wheat were used for the chromosomal assignment of the *CENH3* copies in *S. cereale*. Each of the seven wheat-rye addition lines 'Chinese Spring'/'Imperial' (2n=44 (42+2R)) (Driscoll and Sears 1971) contains the entire hexaploid wheat *Triticum aestivum* genome from 'Chinese Spring' and a pair of rye *S. cereale* chromosomes from 'Imperial'. A comparison of the sequences of the alpha variants of *CENH3* cDNA between 'Imperial' and 'Chinese Spring' revealed a high (99%) level of identity and it is no wonder why the consensus amino acid sequences of 'Imperial' αCENH3-v1 and 'Chinese Spring' αCENH3 were found to be identical (Fig. 1A). The 100% amino acid sequence identity between rye and wheat prevents this variant from being assigned to particular rye chromosomes using addition lines. Several positions in these sequences are polymorphic. The most frequently observed polymorphism is at position 33 of the NTT domain (framed in Fig. 1A), where glutamine acid instead of asparagine acid is present in 20% of the 'Imperial' clones and in 47% of the 'Chinese Spring' clones.

Sixteen percent of the cDNA clones of the alpha variants of 'Imperial' *CENH3* have a 9 bp deletion and represent the minor variant, α*CENH3-v2*, according to our classification (Fig. 1A). None of the 19 'Chinese Spring' clones was found to have this deletion, allowing us to use addition lines for assigning this variant of *CENH3* to rye chromosomes. There was only one addition line (that with rye chromosome 6R), whose clones (39%) had this deletion (Fig. 2). None of the other addition lines had any clone with this deletion.

The shorter, 456-bp-long forms of 'Imperial' *CENH3* DNA produce protein molecules, each containing 156 amino acids and collectively denoted as βCENH3-v1

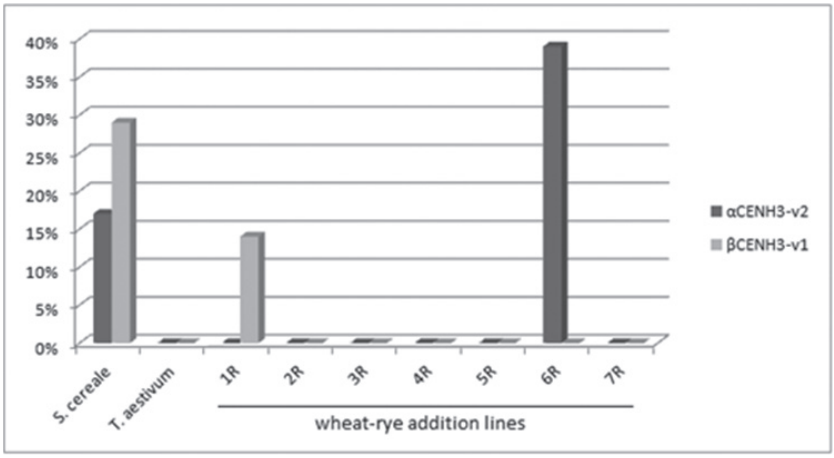

Figure 2. Histogram showing the percentage of α*CENH3*-v2 and β*CENH3*-v1 in the cDNA clones from rye *S. cereale* (cv. Imperial), wheat *T. aestivum* (cv. Chinese Spring) and wheat-rye (1R–7R) addition lines.

(Fig. 1B). They are less common than α*CENH3* and make up 29% of the clones. These sequences did not occur from 'Chinese Spring' DNA amplification and thus their chromosomal assignment using addition lines was possible. There was only one addition line (that with rye chromosome 1R), whose clones (specifically, 14%) were found to have sequences with this structure and of this size (Fig. 2). 'Chinese Spring' cDNA became the source of *CENH3* sequences, each containing a 6-bp insertion in the NTT domain and corresponding, according to our classification, to β*CENH3*-v2 in 'Imperial' DNA. The nucleotide sequence homology of this variant between wheat and rye is 100% and thus addition lines were of as little help in assigning this variant to particular rye as they were with α*CENH3-v1*.

Analysis of databases and phylogenetic relationships between *CENH3* in rye and wheats

To confirm the chromosomal assignment of various *CENH3* variants made using addition lines and to assign α*CENH3*-v1 and β*CENH3*-v2 to particular chromosomes, we analyzed entries in the Sequence Read Archive (European Bioinformatics Institute, accession IDERP001745) for sorted rye chromosomes 1R-7R (Martis et al. 2013). Some sequence reads from chromosome 1R (accession ERX140512) were found to have a high nucleotide identity, 87–97%, of the coding sequence of α*CENH3-v1*. That the contig composed of these reads was really α*CENH3-v1* was indicated by the absence of the 9-bp deletion, typical of α*CENH3-v2*, in one of the reads (ERX140512.2250257), which contained the first exon of α*CENH3* entirely and the coding amino acid sequence identical to αCENH3-v1.

Additionally, two of the reads from chromosome 1R were found to contain the coding region (positions 1 through 328) of β*CENH3-v1* (ERX140512.1955393, ERX140512.290111): they had no 6-bp insertion in the NTT domain that all β*CENH3-v2* normally have and they had large deletions in the NTT that delineate beta forms from alpha forms. Some sequence reads from chromosome 6R (accession ERX140517) were found to have a high nucleotide identity to the HFD in β*CENH3*. Because these reads contained only the most conserved region of the HFD (the last 42 amino acids) and because this region was identical between β*CENH3-v1* and β*CENH3-v2*, we were unable to tell these variants from each other, however, one thing was clear: β*CENH3* is located on chromosome 6R. Thus, the analysis of the Sequence Read Archive for gDNA sequences amplified from sorted rye chromosomes 1R-7R assigned α*CENH3-v1* to chromosome 1R, the beta form of *CENH3* to chromosome 6R and confirmed the addition line-based assignment of β*CENH3-v1* to chromosome 1R. In summary, the main forms of *CENH3* (the alpha and beta forms) are located on rye chromosomes 1R and 6R, the commonest variants, α*CENH3-v1* and β*CENH3-v1*, are on chromosome 1R, and the less common α*CENH3-v2* and probably β*CENH3-v2* are on chromosome 6R. It should be noted that analysis of the most recent version of the rye genome without chromosome sorting (Bauer et al. 2017) did not reveal any *CENH3* variants other than those described herein.

A high level of identity of *CENH3* sequences between wheat and rye is not consistent with a wealth of plant species data that suggest considerable between-species differences in the structure of this protein (Sanei et al. 2011, Masonbrink et al. 2014, Dunemann et al. 2014, Maheshwari et al. 2015). This encouraged us to explore the phylogenetic relationships between the different *CENH3* variants in rye, wheat and diploid species seen as putative donors of the hexaploid wheat genomes. As was found out, *T. urartu* accession KM507181 shares a high (99%) nucleotide identity with 'Imperial' α*CENH3-v2*, and *A. tauschii* accession KM507186, 100% identity with 'Imperial' β*CENH3-v2* (Fig. 1A, B). The neighbor-joining phylogenetic tree for CENH3 amino acid sequences consists of two major clusters, one with alpha forms and another with beta forms (Fig. 3). The topology of the clusters displays some differences in the ways they branch. The bootstrap values in the beta cluster suggest its clearer partitioning into subclusters. In the alpha cluster, CENH3's from *T. urartu* and *A. speltoides* share the same subcluster, while in the beta cluster, each of these species forms a separate branch. CENH3 sequences obtained from the lines, in which added chromosomes (particularly, 1R and 6R) were found to carry rye *CENH3* copies, share the same subcluster with the 'Imperial' sequences. The major variants (variants 1) form prominent subclusters, while the minor variants (variants 2) lie closer to the diploid species *T. urartu, A. tauschii* and *A. speltoides*. Although these species are considered the main contributors to the hexaploid wheat genome, the level of CENH3 identity between them and the rye cultivar Imperial is just as high.

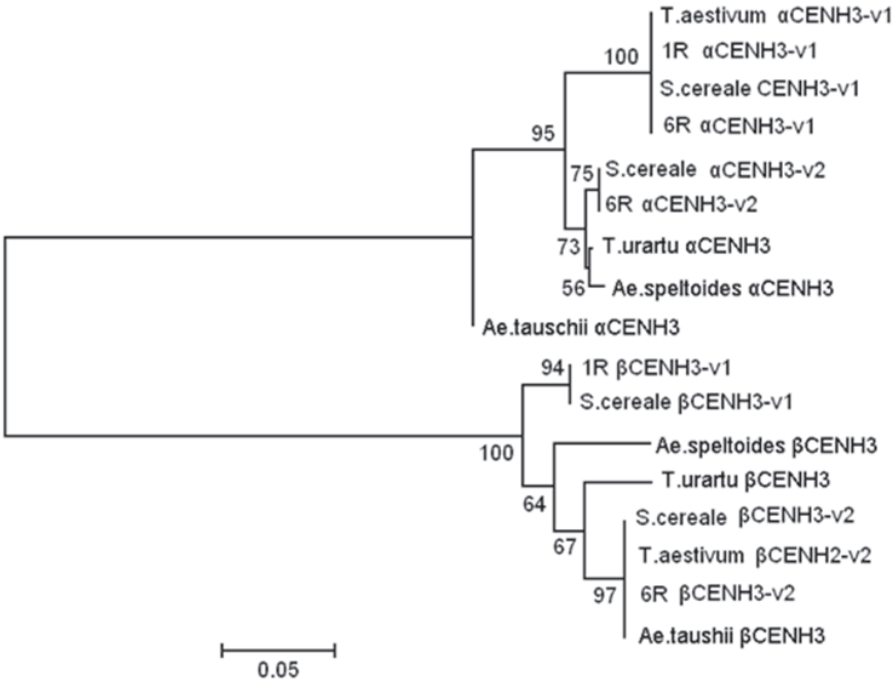

Figure 3. Phylogenetic tree of the CENH3 proteins. Phylogenetic tree inferred using JTT+G models (measures distances) and bootstrapping (1000 replicates). Bootstrap values are indicated on the branches. Rye *S. cereale* (cv. Imperial), wheat *T. aestivum* (cv. Chinese Spring) and wheat-rye (1R–7R) addition lines. NCBI accessions are: αCENH3 and βCENH3 in *T. urartu* (KM507181, KM507184), *Ae. tauschii* (KM507183, KM507186), *Ae. speltoides* (KM507182, KM507185). The scale bar is substitutions per site.

Discussion

Most of the 67 plant species with the CENH3 sequence publicly available – including those that have undergone whole-genome duplication – have this essential gene as a singleton (Maheshwari et al. 2015). Note that sampling bias is a factor here, for 23 species (1/3) were from Brassicaceae. In cereals, three CENH3 variants described in the oats *Avena sativa* Linnaeus, 1753 (Ishii et al. 2015) represent the alpha form and two forms of CENH3 appear for the first time in the closest rye relatives: the Triticeae genera barley, wheat and *Aegilops* (Sanei et al. 2011, Yuan et al. 2015). Comparative analyses of the cereal genomes revealed a whole-genome duplication (WGD), which occurred between 53 and 94 MYA (i.e. before the divergence of the cereal genomes), a recent segmental duplication between chromosomes 11 and 12 and numerous individual gene duplications (Feuillet and Salse 2009). Because the Triticeae species carry a haploid set of seven chromosomes, it is hypothesized that the two forms of CENH3 emerged from a duplication event (Yuan et al. 2015), which in this case is most likely to represent a local duplication event that had taken place before the barley and wheat-rye lineages split.

The now commonly accepted viewpoint authored by Ohno (1970) is that a local duplication event gives rise to two functionally redundant, paralogous genes, one of which will carry on under attenuated selective pressure and will thus be freed from selective constraints. As a result, each copy as this starts to accumulate mutations that would have been deleterious to the gene, if it were a singleton. In reference to *CENH3* in rye, wheat and barley, it can be hypothesized that the copies accumulating deleterious mutations are the beta forms, because their NTT domains contain several deletions of various size that the NTT domains of the alpha forms do not. Interestingly, inactivation of beta CENH3 in barley did not result in a major phenotype and point mutation impairs centromeric *CENH3* loading and induces haploid plants (Karimi-Ashtiyani et al. 2015). The structure of *CENH3* in cereal species that have this gene as a singleton (for example, the rice *O. sativa*) is much closer to the structure of the Triticeae alpha forms (74% identity, while no alignment with the beta form is possible because of extended deletions), which can be regarded as being evolutionarily more ancient than beta forms. According to the theory of evolution by gene duplication (Ohno 1970, Wagner 2002), deleterious mutations should accumulate and lead to a rapid loss of one of the paralogs; however, this is not the case with *CENH3* copies in Triticeae. Among the possible reasons for the observed inconsistency with the theory is the acquisition of new functions by a duplicate gene or by both copies or sub- or neofunctionalization (Conant and Wolfe 2008). For example, in wild tetraploids of wheat, β*CENH3* has a much lower expression level than α*CENH3*, while in cultivated tetraploids β*CENH3* transcripts are enhanced to near α*CENH3* level (Yuan et al. 2015). All the *CENH3* variants that we have described have been found in rye cDNA, that is, they are transcribed; however, to understand the function of each particular variant, special research is required.

According to our results, both main forms, α*CENH3-v1* and β*CENH3-v1*, are located on rye chromosome 1R. Obviously, these copies do not reside there next to each other, but are somewhat spaced. There are two facts that support this statement. First, the two-copy organization of *CENH3* makes these copies very likely to end up with gene conversion events that homogenize their sequences (Wagner 2002). The presence of a large deletion in β*CENH3* and the heterogeneity of the nucleotide and amino acid sequences of the alpha and beta forms suggest quite the opposite. Secondly, analyzing *T. urartu* genome sequencing data (Ling et al. 2013), we found that α*CENH3* and β*CENH3* are 24 kb apart (locus KD187944.1, unplaced genomic scaffold 30245, BioProject PRJNA182347). Considering a high level of identity of *CENH3* sequences between rye and *T. urartu*, it is possible that the genomic region containing *CENH3* has remained highly syntenic between the rye and wheat genomes.

Various molecular mechanisms have been proposed to explain the emergence of these spaced gene copies on the same or different chromosomes (Glover et al. 2015). Some of these mechanisms are 1) retroposition where genes are reverse transcribed and reinserted back into the genome; 2) ectopic recombination during double-strand break (DSB) repair; 3) transposable element mediated gene captured and moved throughout plant genomes; 4) exon shuffling. All these processes involve rearrangements in gene structure. However, the amount of data available so far is insufficient for making an unbiased judgement as to which of these mechanisms should be accepted.

A comparison of the genetic maps between rye and barley shows that rye chromosome 1R and barley chromosome 1H are fully collinear (Martis et al. 2013). Noteworthy, 1R is the only rye chromosome in which no large translocations have been found as the rye karyotype was being shaped from a Triticeae progenitor (Martis et al. 2013). It has previously been established that *CENH3* is located on barley chromosome 1H (Sanei et al. 2011). In wheat, three highly homologous *CENH3* genes have been assigned to chromosomes 1A, 1B and 1D by PCR amplification in nullisomic-tetrasomic lines (Li et al. 2013) and fluorescent in situ hybridization (Yuan et al. 2015). Thus, the presence of *CENH3* genes on chromosome 1 is a shared feature of Triticeae genera. The barley species *H. vulgare* and *H. bulbosum* have additionally been found to have β*CENH3* on chromosome 6H (Sanei et al. 2011). Taking together the existing data and our assignment of *CENH3* copies to rye chromosome 6R, it can be stated that, the *CENH3* genes appeared on chromosome 6 as they did on chromosome 1 in a Triticeae species before the barley and wheat-rye lineages split.

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Advances in cytogenetics of Brazilian rodents: cytotaxonomy, chromosome evolution and new karyotypic data

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Abstract

Rodents constitute one of the most diversified mammalian orders. Due to the morphological similarity in many of the groups, their taxonomy is controversial. Karyotype information proved to be an important tool for distinguishing some species because some of them are species-specific. Additionally, rodents can be an excellent model for chromosome evolution studies since many rearrangements have been described in this group.This work brings a review of cytogenetic data of Brazilian rodents, with information about diploid and fundamental numbers, polymorphisms, and geographical distribution. We point out that, even with the recent efforts on cytogenetic studies in this group, many species lack karyotypic data. Moreover, we describe for the first time the karyotype of *Carterodon sulcidens* (Lund, 1838) (Family Echimyidae), a new fundamental number for an undescribed species of *Neacomys* Thomas, 1900 (Family Cricetidae, Subfamily Sigmodontinae), and illustrate the karyotype of a Brazilian specimen of *Mus musculus* Linnaeus, 1758 (Family Muridae). This review compiles the cytogenetic data on Brazilian rodents reported in the last three decades, after the last revision published in 1984, including synonyms, chromosomal variations, and geographic distribution. Additionally, it also reinforces that Brazilian biodiversity is still poorly known, considering the new data reported here.

Keywords
Chromosomes, Rodentia, karyotype evolution, Carterodon sulcidens, Neacomys

Introduction

More than three decades after the last revision of cytogenetics of Brazilian rodents (Kasahara and Yonenaga-Yassuda 1984), in which the karyotypes of approximately 60 species were reported, several new karyotypes and chromosomal rearrangements have been described. In the last 30 years, huge progress has been made, and up to this date, new species have frequently been described. However, as we shall explore herein, there still remain gaps in knowledge about many species.

Cytogenetic information on Brazilian rodents was firstly described by Cestari and Imada (1968) for the species referred to as *Akodon arviculoides cursor* Thomas, 1913. From then on, cytogenetic data confirmed the great chromosomal variability in rodents, especially after the advent of banding techniques in the beginning of the 1970s.

Throughout the following decades, several Master dissertations and PhD theses have addressed cytogenetic studies on Brazilian rodents. It became evident that karyotypic data could contribute to accurate taxonomic information, since different names were applied to groups that shared the same karyotype, and very distinct karyotypes were attributed to a single species. Additionally, major fieldwork efforts in Brazil (especially in unexplored areas) have led to the discovery of many new species.

The increasing number of cytogenetic studies on rodents resulted in the characterization of banding patterns, recognition of sex chromosomes, identification of supernumerary chromosomes, pericentric inversions and Robertsonian rearrangements, variations in the amount and localization of constitutive heterochromatin, and recognition of species (cytotaxonomy). These discoveries have led researchers to consider that rodents have undergone a "karyotypic explosion" process and that they stand out as an excellent group for chromosomal evolution studies, since they present many examples of chromosome rearrangements. These rearrangements may have played an important role in karyotype diversification and speciation, with the reduction of gene flow due to meiotic problems (King 1993, Rieseberg 2001, Patton 2004, Faria and Navarro 2010).

Previously, chromosome evolution studies were essentially based on the comparison of banding patterns (Yonenaga-Yassuda et al. 1975, 1987a, Leal-Mesquita et al. 1992, Silva and Yonenaga-Yassuda 1999). Later, the association of cytogenetics with molecular biology allowed for a new important approach for studying karyotype evolution. Notwithstanding, molecular cytogenetics allows the localization of specific DNA sequences in the chromosomes based on DNA denaturation and its subsequent annealing with complementary sequences. In Brazilian rodents, localization of specific sequences using fluorescence *in situ* hybridization (FISH) was specifically applied in the Akodontini and Oryzomyini tribes of the Family Cricetidae, Subfamily Sigmodontinae, which is traditionally divided into 10 tribes and one *incertae sedis* group (Pardiñas et al. 2015a). Nevertheless, this kind of approach is still lacking for the other

tribes of Sigmodontinae, and the remaining rodent families, mainly because of the difficulty in obtaining specific probes.

FISH was first performed using telomeric sequence probes, revealing that, besides the telomeric position itself, the sequences could also be detected at telomeric interstitial sites (ITS), such as those present in the Sigmodontinae genus *Akodon* Meyen, 1833, *Thaptomys* Thomas, 1916, and *Cerradomys* Weksler, Percequillo & Voss, 2006 (Fagundes et al. 1997a, Fagundes and Yonenaga-Yassuda 1998, Silva and Yonenaga-Yassuda 1998a, Andrades-Miranda et al. 2002a, Ventura et al. 2004, 2006). These ITS were correlated with components of constitutive heterochromatin, amplification of $TTAGGG_n$ sequences, telomeres remnants after chromosomal rearrangements or reservoirs for future fission rearrangements. On the other hand, the absence of ITS in other Sigmodontinae species with chromosome polymorphisms, such as *Oligoryzomys* Bangs, 1900, and *Rhipidomys* Tschudi, 1845, was also described (Silva and Yonenaga-Yassuda 1997, 1999).

More recently, probes from entire chromosomes were obtained by microdissection or flow sorting, representing a breakthrough in evolutionary studies. The first Brazilian study employing this technique was published by Fagundes et al. (1997b), in which the largest pair (pair 1) of the karyotype of the rodent *Akodon cursor* (Winge, 1887) (Subfamily Sigmodontinae, tribe Akodontini) was obtained in order to investigate regions of homology between chromosomes of this species and *Akodon montensis* Thomas, 1913.

More than one decade later, Hass et al. (2008), using *Mus musculus* commercial chromosome probes, established chromosomal homology maps between five species of the tribe Akodontini, plus one Oryzomyini species. One year later, Ventura et al. (2009) performed chromosome painting using *Akodon* species-specific probes.

After the tribe Akodontini, Oryzomyini is the second most studied tribe by chromosome painting from the Subfamily Sigmodontinae. Comparisons between *Hylaeamys megacephalus* (G. Fischer, 1814) and *Cerradomys langguthi* Percequillo, Hingst-Zaher & Bonvicino, 2008 were performed by Nagamachi et al. (2013), and Di-Nizo et al. (2015) studied chromosome evolution within the genus *Oligoryzomys*. In addition, chromosome painting using *Hylaeamys megacephalus* probes was performed to compare the Akodontini and Oryzomyini tribes (Suárez et al. 2015, Pereira et al. 2016) and, more recently, two populations of *Oecomys catherinae* Thomas, 1909 were also evaluated (Malcher et al. 2017).

The role of cytogenetics in species recognition (cytotaxonomy) has been know for a while, considering that many rodents' species are morphologically similar (Bonvicino and Weksler 1998, Christoff et al. 2000, Percequillo et al. 2008). In addition, molecular phylogenetics improved the possibility of recognizing monophyletic clades. In fact, the proper identification of undescribed species is only possible with the association of morphology, cytogenetics, geographic distribution and molecular phylogeny. Altogether, these different approaches are essential not only for identifying the cryptic Brazilian biodiversity but also for public health programs, since some rodents' species are Hantavirus reservoirs (Souza et al. 2002, Lemos et al. 2004).

Therefore, the aim of this review is to compile all the cytogenetic data available for Brazilian rodents, presenting not only the diploid and fundamental numbers, but also the chromosomal polymorphisms, synonyms, and geographic distribution. In addition, we describe for the first time the karyotype of the monotypic species *Carterodon sulcidens*, and show the karyotype of Brazilian specimen of the introduced rodent *Mus musculus* for the first time. A new fundamental number for a putative undescribed species of *Neacomys* is also reported. In addition, to investigate phylogenetic relationships among *Neacomys* species, molecular analyses based on the gene cytochrome *b* were performed. This work discusses the most common rearrangements in each group, by pointing out the species which could represent complexes of species (thus needing revision) or present polymorphisms, as well as highlighting the species and families that lack cytogenetic information.

Material and methods

Literature revision

This review was done after an extensive revision of the literature, including Master's and Ph.D. theses, when available (Table 1). Abstracts from congresses and conferences were not considered, since karyotype pictures were only available during the events and access to this kind of material is restricted. Chromosome rearrangements in Table 1 were named as described in the literature (for example Robertsonian rearrangement, centric fusion, etc.). However, in the text, we refer to centric fusion/fission as a synonym of Robertsonian rearrangement (Sumner 2003). Except for the species that have not been formally described (e.g. *Thaptomys* sp., *Proechimys* gr. *goeldii*, etc.), the taxonomical classification follows the one proposed by Patton et al. (2015) and Fabre et al. (2016), that recently included *Myocastor* Kerr, 1792 within the Family Echimyidae.

Sampling

The single female of *Carterodon sulcidens* (lab number: CIT787/ field number: APC58) was captured in Serra da Mesa, State of Goiás, Brazil (13°53'S, 48°19'W), a region characterized by the Cerrado biome. Additionally, five males of *Mus musculus* (field number: PCH4078, 4079, 4094–96) were captured in Guará, São Paulo State, Brazil (20°29'S, 47°51'W), a transitional region between the Cerrado and Atlantic Forest.

Regarding *Neacomys*, four specimens of *N. amoenus amoenus* Thomas, 1903 were captured in Mato Grosso State, Brazil, in a transitional area between Cerrado and Amazonian Rainforest. Two specimens of *Neacomys* sp. were captured, one at Vila Rica (Mato Grosso State), and the other at Igarapé-Açu (Amazonas State), Brazil (field number, locality, and coordinates are presented in Suppl. material 1).

Table 1. Compilation of cytogenetic data of Brazilian rodents, with the respective synonyms, diploid number (2n) and fundamental number (FN), karyotypic variation, localities (according to Bonvicino et al. 2008 and Patton et al. 2015) and references.

		Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Caviidae		*Cavia aperea* Erxleben, 1777	-	64	116, 124	-	PE, SE, AL, BA, MG, GO, MT, MS, MG, SP, PR and SC	Maia 1984, Bonvicino et al. 2008, Gava et al. 2011
		Cavia fulgida Wagler, 1831	-	64	124	-	Eastern Brazil, between MG and SC	Woods and Kilpatrick 2005, Walker et al. 2014
		Cavia intermedia Cherem, Olimpio, and Ximénez, 1999	*Cavia* aff. *magna*	62	108	-	Endemic from SC (Ilhas Moleques do Sul)	Gava et al. 1998, Woods and Kilpatrick 2005
		Cavia magna Ximénez, 1980	-	62; 64	102; 124	Pericentric inversions; addition and deletion of constitutive hetechromatin; Robertsonian rearrangement	RS and SC	Bonvicino et al. 2008, Gava et al. 2011, Walker et al. 2014
		Cavia porcellus (Linnaeus, 1758)	-	64*	100-102	Polymorphism in chromosome 1	All Brazilian States	Bonvicino et al. 2008, Walker et al. 2014
		Galea flavidens (Brandt, 1835)	-	N/A	N/A	-	Northwestern MG and Northeastern GO	Bonvicino et al. 2008
		Galea spixii (Wagler, 1831)	-	64	118	-	PA, MT, MG, BA, PE, PB, RN, CE, PI, MA and DF	Maia 1984, Bonvicino et al. 2008
		Hydrochoerus hydrochaeris (Linnaeus, 1766)	-	66	102	-	All Brazilian States, except CE	Wurster et al. 1971, Bonvicino et al. 2008
		Kerodon acrobata Moojen, 1997	-	52	92	-	Northeastern GO	Bonvicino et al. 2008, Zappes et al. 2014
		Kerodon rupestris (Wied-Neuwied, 1820)	-	52	92, 94	Pericentric inversion	From PI and CE to Northern MG	Maia 1984, Bonvicino et al. 2008, Lessa et al. 2013
Family Cricetidae - Subfamily Sigmodontinae	Tribe Akodontini	*Akodon azarae* (J. B. Fischer, 1829)	-	37-38	40-44	Variation in the Y morphology; deletion of the X long arm	Southern Brazil	Kasahara and Yonenaga-Yassuda 1984, Vitullo et al. 1986, Sbalqueiro 1989, Pardiñas et al. 2015b
		Akodon cursor (Winge, 1887)	*Akodon arviculoides*	14-16	18-26	Pericentric inversions in pairs 2, 4 and 6; centric fusion and pericentric inversion in pairs 1 and 3; trisomy of the pair 7; ITS	Atlantic Forest formations in Eastern Brazil from PB to PR and Eastern MG	Cestari and Imada 1968, Fagundes et al. 1997a, Fagundes et al. 1997b, Fagundes et al. 1998, Geise et al. 1998
		Akodon lindberghi Hershkovitz, 1990	*Akodon* sp.	42	42	ITS	Cerrado habitat, Central and Southeastern Brazil	Svartman 1989, Svartman and Almeida 1994, Geise 1995
		Akodon montensis Thomas, 1913	*Akodon* aff. *arviculoides*, *Akodon* sp.	23; 24-26; 24/25; 23/24	40; 42; 44	X monosomy; 1 or 2 B chromosomes; mosaicism; reciprocal translocation (1, 6); sex chromosome heteromorphism	Southeastern Brazil, from RJ to RS, including gallery Forest settings in MG and GO	Geise et al. 1998, Fagundes et al. 1997b, Fagundes et al. 2000

		Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Cricetidae - Subfamily Sigmodontinae	Tribe Akodontini	*Akodon mystax* Hershkovitz, 1998	-	42, 44	42	-	Pico da Bandeira, in the border of MG and ES	Musser and Carleton 2005, Gonçalves et al. 2007, Pardiñas et al. 2015b
		Akodon paranaensis Christoff, Fagundes, Sbalqueiro, Mattevi and Yonenaga- Yassuda, 2000	*Akodon serrensis*	44	44	Non-disjunction of the sex chromosomes (2n = 43 and 45)	Eastern RJ and SP and Southern Brazil	Christoff et al. 2000
		Akodon reigi E. M. González, Langguth & Oliveira, 1998	-	44	44	-	Southernmost Brazil (RS)	Musser and Carleton 2005
		Akodon sanctipaulensis Hershkovitz, 1990	-	N/A	N/A	-	Serra do Mar, Southeastern Brazil	Musser and Carleton 2005
		Akodon sp. n.	-	9; 10	14-16	X monosomy; pericentric inversion in pair 3; ITS	Only known from its type locality, MT	Silva and Yonenaga-Yassuda 1998a
		Akodon toba Thomas, 1921	*Akodon varius*	40*; 42-43*	40*; 44*	Karyotype of specimens from Paraguay	Southwestern MS	Bonvicino et al. 2008, Pardinãs et al. 2015a
		Bibimys labiosus (Winge, 1887)	-	70	80	-	Northern RS, and Southeastern MG and RJ	Bonvicino et al. 2008, Gonçalves et al. 2007
		Blarinomys breviceps (Winge, 1887)	-	28; 31 (29+2Bs); 34; 37 (36 + 1B); 43 (39 + 4Bs); 45 (44 + 1B); 52; 52 (50 + 2Bs)	48, 50; 50; 50; 50; 50; 50, 51; 50; 50	B chromosomes; Robertsonian rearrangement; ITS	Atlantic Forest regions of Southeastern Brazil (from BA to SP, and Eastern MG)	Silva et al. 2003, Musser and Carleton 2005, Geise et al. 2008, Ventura et al. 2012
		Brucepattersonius griserufescens Hershkovitz, 1998	-	52	52, 53	Pericentric inversion in pair 2	Eastern MG, and ES to RJ	Bonvicino et al. 1998a, Musser and Carleton 2005
		Brucepattersonius igniventris Hershkovitz, 1998	-	N/A	N/A	-	Southeastern SP	Musser and Carleton 2005, Bonvicino et al. 2008, Rossi 2011
		Brucepattersonius iheringi (Thomas, 1896)	*Oxymycterus iheringi*	52	52	-	Southern Brazil	Musser and Carleton 2005, Vilela 2005
		Brucepattersonius soricinus Hershkovitz, 1998	-	52	52	-	Eastern SP and PR	Musser and Carleton 2005, Di-Nizo et al. 2014
		Castoria angustidens (Thomas, 1902)	*Akodon* sp., *A. leucogula*, *A. serrensis*	46	46	ITS	Atlantic Forest from Southeastern ES to RS	Geise et al. 1998, Christoff et al. 2000, Abreu et al. 2014, Pardiñas et al. 2015b, Pardiñas et al. 2016a
		Deltamys araucaria Quintela, Bertuol, González, Cordeiro-Estrela, Freitas, Gonçalves, 2017	-	34	34	-	Only known from its type locality, São Francisco de Paula/ RS	Quintela et al. 2017
		Deltamys kempi Thomas, 1917	-	35-38	38	Centric fusion/fission; multiple sex determination system.	Eastern RS	Sbalqueiro et al. 1984, Castro et al. 1991, Musser and Carleton 2005, Bonvicino et al. 2008

		Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Cricetidae - Subfamily Sigmodontinae	**Tribe Akodontini**	*Deltamys* sp.	-	40	40	-	Esmeralda (RS)	Ventura et al. 2011
		Gyldenstolpia fronto Winge, 1887	*Kunsia fronto*	N/A	N/A	-	Lagoa Santa (MG)	Musser and Carleton 2005, Pardiñas et al. 2008, Pardiñas and Bezerra 2015
		Gyldenstolpia planaltensis (Avila-Pires, 1972)	*Kunsia fronto planaltensis*	N/A	N/A	-	Westcentral Brazil	Pardiñas and Bezerra 2015
		Juscelinomys candango Moojen, 1965	-	N/A	N/A	-	DF	Musser and Carleton 2005
		Kunsia tormentosus (Lichtenstein, 1830)	-	44	42	-	Westcentral Brazil	Andrades-Miranda et al. 1999, Musser and Carleton 2005
		Necromys lasiurus (Lund, 1840)	*Zygodontomys lasiurus*, *Bolomys lasiurus*	34, 33, 33/34	34	Robertsonian rearrangement; centric fusion, X polymorphism; mosaicism (XX/X0)	Southern Amazon River, Brazil	Maia and Langguth 1981, Kasahara and Yonenaga-Yassuda 1983, Svartman and Almeida 1993a, Musser and Carleton 2005
		Necromys urichi (J. A. Allen & Chapman, 1897)	-	18	30	-	Northern Brazil	Reig et al. 1986, Musser and Carleton 2005
		Oxymycterus amazonicus Hershkovitz, 1994	-	54	N/A	-	Lower Amazon Basin, Southern Amazon River, between Tocantins and Madeira Rivers, Central Brazil, Northwestern MT	Bonvicino et al. 1998a, Musser and Carleton 2005
		Oxymycterus caparoae Hershkovitz, 1998	-	54	64	-	Eastern MG and ES to RJ	Bonvicino et al. 1998a, Musser and Carleton 2005
		Oxymycterus dasytrichus (Schinz, 1821)	*Oxymycterus angularis*, *O. hispidus*, *O. roberti*	54	64	-	Atlantic and interior forest of Eastern Brazil (PE, AL, SE, BA, MG, ES, RJ, SP and PA)	Musser and Carleton 2005, Moreira et al. 2009
		Oxymycterus delator Thomas, 1903	*Oxymycterus* sp., *Oxymycterus roberti*	54	62	-	Southcentral Brazil	Svartman and Almeida 1993b , Bonvicino et al. 2005a, Musser and Carleton 2005
		Oxymycterus inca Thomas, 1900	-	54	N/A	-	Acre	Bonvicino et al. 1998a, Bonvicino et al. 2008
		Oxymycterus nasutus (Waterhouse, 1837)	-	54	64	-	Eastern RS to Eastern SP	Musser and Carleton 2005, Quintela et al. 2012
		Oxymycterus quaestor Thomas, 1903	*Oxymycterus judex*	54	N/A	-	Eastern Brazil, from RS to SP, and Serra dos Órgãos (RJ)	Bonvicino et al. 1998a, Bonvicino et al. 2008, Oliveira and Gonçalves 2015
		Oxymycterus rufus (G. Fischer, 1814)	-	54	66	-	Southeastern MG	Geise 1995
		Podoxymys roraimae Anthony, 1929	-	16	26	-	RR	Pérez-Zapata et al. 1992, Musser and Carleton 2005, Bonvicino et al. 2008

		Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Cricetidae - Subfamily Sigmodontinae	Tribe Akodontini	*Scapteromys aquaticus* Thomas, 1920	-	32	40	-	Westernmost RS	Bonvicino et al. 2013
		Scapteromys meridionalis Quintela, Gonçalves, Althoff, Sbalqueiro, Oliveira, Freitas, 2014	*Scapteromys* sp. 1, *Scapteromys* sp. 2	34, 36	40	Centric fusion	Southern Brazil	Freitas et al. 1984, Bonvicino et al. 2013, Quintela et al. 2014
		Scapteromys tumidus (Waterhouse, 1837)	-	24	40	-	Southernmost Brazil (RS)	Brum-Zorrilla et al. 1986, Musser and Carleton 2005
		Thalpomys cerradensis Hershkovitz, 1990	-	36	34	-	Cerrado of Central Brazil	Andrade et al. 2004, Musser and Carleton 2005
		Thalpomys lasiotis Thomas, 1916	*Akodon reinhardti*	37, 38	38	Centric fusion/fission; heterochromatin variation in an autosomal pair	Cerrado of Central Brazil	Yonenaga-Yassuda et al. 1987b
		Thaptomys nigrita (Lichtenstein, 1829)	*Akodon* (*Thaptomys*) *nigrita*	52	52	-	Southeastern Brazil, BA to RS	Yonenaga 1972, Yonenaga 1975, Souza 1981, Castro 1989, Fagundes 1993, Geise 1995
		Thaptomys sp.	-	50	48	ITS	Only known from its type locality - BA	Ventura et al. 2004
	Tribe Ichthyomyini	*Neusticomys ferreirai* Percequillo, Carmignotto & Silva, 2005	-	92	98	-	Amazonian lowland of MT and PA	Percequillo et al. 2005
		Neusticomys oyapocki (Dubost & Petter, 1979)	-	N/A	N/A	-	Amazonian of Northern Brazil (AP and PA)	Voss 2015a
	Tribe Oryzomyini	*Cerradomys akroai* Bonvicino, Casado & Weksler, 2014	-	60	74	-	TO	Bonvicino et al. 2014
		Cerradomys goytaca Tavares, Pessôa & Gonçalves, 2011	-	54	62, 63, 66	Different interpretation of morphology of small pairs and pericentric inversion in small chromosome	Northeastern littoral of RJ and Southern littoral of ES (Restinga region)	Tavares et al. 2011, Bonvicino et al. 2014
		Cerradomys langguthi Percequillo, Hingst- Zaher, and Bonvicino, 2008	*Oryzomys* sp. B	46, 48, 49, 50	56	Centric fusion/ fission; Y polymorphism; ITS	PE, MA, PB and CE	Maia and Hulak 1981, Percequillo et al. 2008, Nagamachi et al. 2013
		Cerradomys maracajuensis (Langguth & Bonvicino, 2002)	-	56	58	-	Central MT and MS	Langguth and Bonvicino 2002, Bonvicino et al. 2008, Bonvicino et al. 2014
		Cerradomys marinhus (Bonvicino, 2003)	-	56	54	-	GO and Southeatern BA	Bonvicino 2003, Bonvicino et al. 2008
		Cerradomys scotti (Langguth & Bonvicino, 2002)	*Oryzomys* gr. *subflavus*	58	70-72	Pericentric inversion in small chromosome pair; X and Y polymorphisms	GO, Southern MT, Southeastern RO, Northern MS, Western MG and BA, Southeastern TO and Southern PI	Langguth and Bonvicino 2002, Bonvicino et al. 2008

		Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Cricetidae - Subfamily Sigmodontinae	**Tribe Oryzomyini**	*Cerradomys subflavus* (Wagner, 1842)	-	54; 55; 56	62; 63; 64	Robertsonian rearrangement; pericentric inversion in pair 5; X and Y polymorphisms; ITS	PB, PE, AL, BA, MG and SP	Almeida and Yonenaga-Yassuda 1985, Bonvicino et al. 2008
		Cerradomys vivoi Percequillo, Hingst- Zaher & Bonvicino, 2008	*Oryzomys* gr. *subflavus*	50	62, 63	Pericentric inversion; ITS	MG, BA and SE	Andrades-Miranda et al. 2002a, Percequillo et al. 2008
		Drymoreomys albimaculatus Percequillo, Weksler & Costa, 2011	-	62	62	ITS	Atlantic Forest of SP	Percequillo et al. 2011, Suárez-Villota et al. 2013
		Euryoryzomys emmonsae (Musser, Carleton, Brothers & Gardner, 1998)	*Oryzomys emmonsae*	80	86	-	Centraleastern PA	Musser et al. 1998, Bonvicino et al. 2008
		Euryoryzomys lamia (Thomas, 1901)	-	58; 60, 64	82, 84; 84	One name with different karyotypes associated	Western MG and Eastern GO	Bonvicino et al. 1998b, Andrades-Miranda et al. 2000, Bonvicino et al. 2008
		Euryoryzomys macconnelli (Thomas, 1910)	*Oryzomys macconnelli*	64; 58	70; 90	One name with different karyotypes associated	Northern Brazil	Patton et al. 2000, Bonvicino et al. 2008
		Euryoryzomys nitidus (Thomas, 1884)	*Oryzomys nitidus*	80	86	-	AC, RO, Western MT and Southern AM	Patton et al. 2000, Bonvicino et al. 2008
		Euryoryzomys russatus (Wagner, 1848)	*Oryzomys capito*, *O. nitidus*, *O. intermedius*, *O. russatus*	80; 80/81	86	Dissociation of the X chromosome; X and Y polymorphisms	Southeastern Brazil from BA to RS	Yonenaga et al. 1976, Almeida 1980, Zanchin 1988, Silva 1994, Geise 1995, Musser and Carleton 2005, Bonvicino et al. 2008
		Euryoryzomys sp.	-	76	86	-	Only known from its type locality - CE	Silva et al. 2000
		Holochilus brasiliensis(Desmarest, 1819)	-	55; 56-58	56	Centric fusion; 0 to 2 B chromosomes	Southern and Southeastern Brazil	Freitas et al. 1983, Yonenaga-Yassuda et al. 1987a, Bonvicino et al. 2008
		Holochilus chacarius Thomas, 1906	-	48-56*	56-60*	Centric fusion, inversion and B chromosomes	Western MS	Vidal et al. 1976, Bonvicino et al. 2008, Gonçalves et al. 2015
		Holochilus sciureus Wagner, 1842	*Holochilus brasiliensis*	55-56	56	Centric fusion and heteromorphism in pair 1	Northern, Northeastern and Central Brazil	Freitas et al. 1983, Patton et al. 2000, Bonvicino et al. 2008
		Holochilus vulpinus (Brants, 1827)	*Holochilus brasiliensis vulpinus*	40	56	-	Western RS	Freitas et al. 1983, Bonvicino et al. 2008
		Hylaeamys laticeps (Lund, 1840)	*Oryzomys capito*, *O. c. laticeps*, *O. megacephalus*, *Hylaeamys laticeps*	48	60	-	Eastern Atlantic Forest, from BA to Northern RJ	Percequillo 2015b

		Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Cricetidae - Subfamily Sigmodontinae	Tribe Oryzomyini	*Hylaeamys megacephalus* (G. Fischer, 1814)	*Oryzomys capito*, *O. c. laticeps*, *O. megacephalus*;	54	62	-	Northern and Central Brazil	Musser et al. 1998, Patton et al. 2000, Musser and Carleton 2005
		Hylaeamys oniscus (Thomas, 1904)	*Oryzomys capito oniscus*	52	62	-	Northern Rio São Francisco, in PB, PE and AL	Maia 1990, Brennand et al. 2013
		Hylaeamys perenensis (J. A. Allen, 1901)	*Oryzomys perenensis*	52	62	-	Western Brazil	Patton et al. 2000, Bonvicino et al. 2008
		Hylaeamys seuanezi (Weksler, Geise & Cerqueira, 1999)	*Oryzomys capito*, *O c. oniscus*, *O. laticeps*	48	60	-	Southern Rio São Francisco, from BA to RJ	Brennand et al. 2013
		Hylaeamys yunganus (Thomas, 1902)	*Oryzomys yunganus*	52-60	62-67	Chromosome polymorphisms within and between western and eastern population	Northern Brazil	Musser et al. 1998, Patton et al. 2000, Bonvicino et al. 2008
		Lundomys molitor (Winge, 1887)	*Holochilus magnus*	52	58	Variation in the X chromosome	Central RS	Freitas 1980, Freitas et al. 1983, Bonvicino et al. 2008
		Microakodontomys transitorius Hershkovitz, 1993	-	38	46	-	DF	Musser and Carleton 2005, Bonvicino et al. 2008, Paresque and Hanson 2015
		Neacomys amoenus amoenus Thomas, 1903	*Neacomys spinosos amoenus*	64	68	-	Northwestern Brazil	Patton et al. 2000, Bonvicino et al. 2008, Hurtado and Pacheco 2017
		Neacomys dubosti Voss, Lunde & Simmons, 2001	-	62, 64	68	Robertsonian rearrangement	Northern AP	Voss et al. 2001, Musser and Carleton 2005, Bonvicino et al. 2008, Silva et al. 2015
		Neacomys guianae Thomas, 1905	-	56	N/A	-	Northern Brazil	Musser and Carleton 2005, Silva et al. 2015
		Neacomys minutus Patton, da Silva & Malcolm, 2000	-	35-36	40	Robertsonian rearrangement	Southwestern AM	Patton et al. 2000, Bonvicino et al. 2008
		Neacomys musseri Patton, da Silva & Malcolm, 2000	-	34	64-68	Pericentric inversion	Westernmost AC	Patton et al. 2000, Musser and Carleton 2005, Bonvicino et al. 2008
		Neacomys paracou Voss, Lunde & Simmons, 2001	-	56	62, 66	Pericentric inversion	Northernmost Brazil	Voss et al. 2001, Bonvicino et al. 2008, Silva et al. 2015
		Neacomys sp.	-	58	64, 66, 70	Differences in amount of heterochromatin, pericentric inversion	PA and MT	Silva et al. 2015, present study
		Nectomys apicalis Peters, 1861	-	42	40	-	Westernmost Brazil, AC and AM	Patton et al. 2000, Musser and Carleton 2005
		Nectomys rattus Pelzeln, 1883	*Nectomys squamipes*, *N. mattensis*	52-55	52, 54, 56	B chromosomes; X and Y polymorphisms	Northern, Northeastern and Central Brazil	Furtado 1981, Maia et al. 1984, Yonenaga-Yassuda et al. 1988, Zanchin 1988, Svartman 1989, Bonvicino 1994, Bonvicino et al. 1996, Silva and Yonenaga-Yassuda 1998b, Silva 1999, Lima-Rosa et al. 2000, Patton et al. 2000, Bonvicino et al. 2008

		Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Cricetidae - Subfamily Sigmodontinae	**Tribe Oryzomyini**	*Nectomys squamipes* Brants, 1827	-	56-59; 55; 56/57	56-58; 60; 62	B chromosomes; fusion/ fission of autosomes; X monossomy; X and Y polymorphisms	Southeastern Brazil from PE to Northern RS	Yonenaga 1972, Yonenaga et al. 1976, Freitas 1980, Furtado 1981, Maia et al. 1984, Yonenaga-Yassuda et al. 1988, Zanchin 1988, Silva 1994, Geise 1995, Bonvicino et al. 1996, Silva 1999, Bonvicino et al. 2008
		Oecomys auyantepui Tate, 1939	-	64; 66; 72	110; 114; 80	One name with different karyotypes associated	Northern AP and PA	Bonvicino et al. 2008, Lira 2012, Gomes Jr. et al. 2016
		Oecomys bahiensis (Hershkovitz, 1960)	*Oecomys concolor bahiensis*	60	62	-	BA, PE (uncertain distribution)	Langguth et al. 2005, Flores 2010, Gomes Jr. et al. 2016
		Oecomys bicolor (Tomes, 1860)	-	80	140; 142	-	Northern and Central Brazil	Suárez-Villota et al. 2017
		Oecomys catherinae Thomas, 1909	-	60	62; 64	-	Atlantic forest from PB to SC, and Cerrado and Caatinga regions of BA, GO and MG	Musser and Carleton 2005, Bonvicino et al. 2008, Suárez-Villota et al. 2017
		Oecomys cleberi Locks, 1981	-	80; 82	124, 134, 140, 142; 116	One name with different karyotypes associated	DF, PN Emas (GO), and São Joaquim da Barra and Guará (SP)	Lira 2012, Suárez-Villota et al. 2017
		Oecomys concolor (Wagner, 1845)	*Oryzomys* (*Oecomys*) *concolor*	60	62	-	Northwestern Brazil	Furtado 1981, Svartman 1989, Lima-Rosa et al. 2000, Musser and Carleton 2005
		Oecomys franciscorum Pardiñas, Teta, Salazar-Bravo, Myers & Galliari, 2016	-	72	90	-	Pantanal	Pardiñas et al. 2016b, Suárez-Villota et al. 2017
		Oecomys mamorae (Thomas, 1906)	-	N/A	N/A	-	Westcentral Brazil	Musser and Carleton 2005, Suárez-Villota et al. 2017
		Oecomys paricola(Thomas, 1904)	-	68; 70	72; 72, 74, 76	One name with different karyotypes associated	Central Brazil, Southern Amazon River	Musser and Carleton 2005, Suárez-Villota et al. 2017
		Oecomys rex Thomas, 1910	-	62	80	-	Northern Amazon Rio (AP and AM)	Musser and Carleton 2005, Lira 2012, Gomes Jr. et al. 2016
		Oecomys roberti (Thomas, 1904)	-	80; 82	114; 106	-	Amazon region of AM, RO and MT	Musser and Carleton 2005, Patton et al. 2000, Suárez-Villota et al. 2017
		Oecomys rutilus Anthony, 1921	-	54	82, 90	-	Eastern AM	Voss et al. 2001, Gomes Jr. et al. 2016
		Oecomys superans Thomas, 1911	-	80	108	-	Western AM	Patton et al. 2000
		Oecomys trinitatis (J. A. Allen & Chapman, 1893)	-	58	96	-	Northern AC, AM and RR, and Northwestern PA	Bonvicino et al. 2008
		Oecomys sp.	-	86	98	-	AM	Patton et al. 2000, Suárez-Villota et al. 2017
		Oecomys sp.	*Oecomys* cf. *bicolor*	80	124	-	MT	Lima-Rosa et al. 2000, Andrades-Miranda et al. 2001a
		Oecomys sp. 1	-	54	54	-	MT	Suárez-Villota et al. 2017

		Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Cricetidae - Subfamily Sigmodontinae	Tribe Oryzomyini	*Oecomys* sp. 2	-	60	62	-	Aripuanã (MT)	Suárez-Villota et al. 2017
		Oecomys sp. 3	-	60	62	-	São Joaquim da Barra (SP)	Suárez-Villota et al. 2017
		Oecomys sp. 4	-	62	62	-	Vila Rica (MT), Parauapebas (PA)	Suárez-Villota et al. 2017
		Oligoryzomys chacoensis (Myers & Carleton, 1981)	-	58	74	-	Centraleastern Brazil	Myers and Carleton 1981, Bonvicino and Geise 2006
		Oligoryzomys flavescens (Waterhouse, 1837)	-	64-68	66-72	1 to 4 B chromosomes; sex chromosome polymorphisms	Eastern Brazil, from BA to RS	Sbalqueiro et al. 1991, Bonvicino et al. 2008, Di-Nizo 2013
		Oligoryzomys mattogrossae (J. A. Allen, 1916)	*Oligoryzomys eliurus*, *O. fornesi*	62	64-66	Pericentric inversion in small acrocentric pair	DF, Northern MG, GO, BA and Western PE	Bonvicino and Weksler 1998, Andrades-Miranda et al. 2001a, Bonvicino et al. 2008
		Oligoryzomys messorius (Thomas, 1901)	-	66	74	-	Northern Brazil (RO)	Andrades-Miranda et al. 2001a, Weksler and Bonvicino 2015
		Oligoryzomys microtis (J. A. Allen, 1916)	-	64	64, 66	Pericentric inversion in pair 1; X polymorphism	Amazon Basin of Brazil	Aniskin and Voloboeuv 1999, Patton et al. 2000, Musser and Carleton 2005, Di-Nizo et al. 2015
		Oligoryzomys moojeni Weksler & Bonvicino, 2005	*Oligoryzomys* sp.	70	72, 74, 76	Pericentric inversion in small acrocentric pairs; sex chromosome polymorphisms	Southern TO, Northern GO, e Northwestern MG	Lima et al. 2003, Weksler and Bonvicino 2005, Bonvicino et al. 2008, Di-Nizo 2013
		Oligoryzomys nigripes (Olfers, 1818)	*Oligoryzomys delticola*, *O. eliurus*	61, 62	78-82	Pericentric inversions in pairs 2, 3, 4 and 8; Sex chromosome polymorphism; mosaicism (XX/X0)	PB to Northern RS, MG and DF	Almeida and Yonenaga-Yassuda 1991, Paresque et al. 2007, Bonvicino et al. 2008, Di-Nizo 2013
		Oligoryzomys rupestris Weksler & Bonvicino, 2005	*Oligoryzomys* sp. 1	46	52	-	high altitudes in GO and BA	Silva and Yonenaga-Yassuda 1997, Weksler and Bonvicino 2005
		Oligoryzomys stramineus Bonvicino & Weksler, 1998	-	52	68-70	Pericentric inversion in one small acrocentric pair	Cerrado (GO and MG) and Caatinga (PB, PI e PE)	Bonvicino and Weksler 1998, Weksler and Bonvicino 2005
		Oligoryzomys utiaritensis J. A. Allen, 1916	*Oligoryzomys nigripes*	72	76	-	MT and PA (Transition of Cerrado and Amazon)	Agrellos et al. 2012
		Oligoryzomys sp.	*Oligoryzomys* cf. *messorius*	56	58	-	AP	Andrades-Miranda et al. 2001a, Miranda et al. 2008, Weksler and Bonvicino 2015
		Oligoryzomys sp. 2	-	44; 45	52; 53	Mosaicism of a small acrocentric pair; X chromosome polymorphisms	Only known from its type locality (Serra do Cipó, MG)	Silva and Yonenaga-Yassuda 1997
		Pseudoryzomys simplex (Winge, 1887)	-	56	54; 55	Addition of constitutive heterochromatin in pair 17	Central Brazil (MT, TO, GO, MG, SP, BA, AL and PE)	Bonvicino et al. 2008, Moreira et al. 2013
		Scolomys ucayalensis Pacheco, 1991	*Scolomys juruaense*	50	68	-	Westernmost Brazil (AC and AM)	Patton and da Silva 1995, Musser and Carleton 2005, Patton 2015

		Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Cricetidae - Subfamily Sigmodontinae	**Tribe Oryzomyini**	*Sooretamys angouya* (G. Fischer, 1814)	-	57-60	60-64	0-2 B chromosomes	Southeastern Brazil, from ES to RS	Almeida 1980, Zanchin 1988, Silva 1994, Geise 1995, Musser and Carleton 2005, Bonvicino et al. 2008
		Zygodontomys brevicauda (J. A. Allen & Chapman, 1893)	-	86; 84; 82	96-100; 96-98; 94	One name with different karyotypes associated	Northernmost Brazil (AM, RR, PA and AP)	Mattevi et al. 2002, Bonvicino et al. 2009, Voss 2015b
	Tribe Phyllotini	*Calasomys apicalis* Pardiñas, Lessa, Salazar-Bravo & Câmara, 2014	-	62	116	-	Only known in three localities in Central MG	Pardiñas et al. 2014
		Calomys aff. expulsus	-	64	66	-	GO	Mattevi et al. 2005
		Calomys callidus (Thomas, 1916)	-	48	66	-	Western Brazil (RO to MT)	Mattevi et al. 2005, Bonvicino et al. 2010
		Calomys callosus (Rengger, 1830)	-	50	66	-	Western MS	Bonvicino et al. 2008, Bonvicino et al. 2010
		Calomys cerqueirai Bonvicino, Oliveira & Gentile, 2010	-	36; 38	66	Centric Fusion	MG and ES	Bonvicino et al. 2010, Colombi and Fagundes 2014
		Calomys expulsus (Lund, 1840)	-	66	68	-	Caatinga and Cerrado formations from PE to GO	Musser and Carleton 2005, Bonvicino and Almeida 2000
		Calomys laucha (G. Fisher, 1814)	-	64	68	-	Southermost RS	Bonvicino et al. 2008, Mattevi et al. 2005
		Calomys tener (Winge, 1887)	-	64; 66	64; 66	One name with different karyotypes associated	Atlantic Forest region and habitats bordering the Cerrado, Southeastern Brazil (GO, MG, ES, SP, BA and DF)	Bonvicino and Almeida 2000, Mattevi et al. 2005, Musser and Carleton 2005, Bonvicino et al. 2008, Salazar-Bravo 2015
		Calomys tocantinsi Bonvicino, Lima & Almeida, 2003	*Calomys* sp.	46	66	-	Cerrado habitats MT, TO and GO	Bonvicino et al. 2003a, Musser and Carleton 2005, Bonvicino et al. 2008
	Tribe Reithrodontini	*Reithrodon typicus* Waterhouse, 1837	-	28	40	-	Boundary between RS and Uruguay	Freitas et al. 1983, Pardiñas et al. 2015c
	Tribe Sigmodontini	*Sigmodon alstoni* (Thomas, 1881)	-	78, 80, 82*	N/A	Robertsonian polymorphisms; Karyotype of specimens from Venezuela	Northernmost Brazil (RR, AP and PA)	Voss 1992, Bonvicino et al. 2008
	Tribe Thomasomyini	*Rhagomys rufescens* (Thomas, 1886)	-	36	50	-	RJ, SP and MG	Bonvicino et al. 2008, Testoni et al. 2010

		Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Cricetidae - Subfamily Sigmodontinae	Tribe Thomasomyini	*Rhipidomys cariri* Tribe, 2005	*R. cariri baturiteensis*	44	48, 50	FN=50 (type locality), FN=48 (*R. cariri baturiteensis*)	CE, PE and BA	Tribe 2005, Bonvicino et al. 2008, Thomazini 2009,Carvalho et al. 2012, Geise et al. 2010
		Rhipidomys emiliae (J. A. Allen, 1916)	-	44	46, 52, 64	Pericentric inversion	Eastern PA, MT (Serra do Roncador) and Western MA	Silva and Yonenaga-Yassuda 1999, Bonvicino et al. 2008, Tribe 2015
		Rhipidomys gardneri Patton, da Silva & Malcolm, 2000	-	44	50	-	Northwestern AC	Patton et al. 2000, Bonvicino et al. 2008
		Rhipidomys ipukensis R. G. Rocha, Costa & Costa, 2011	-	N/A	N/A	-	Endemic to the Araguaia-Tocantins basin	Rocha et al. 2011, Tribe 2015
		Rhipidomys itoan B. M. de A. Costa, Geise, Pereira and L. P. Costa, 2011	-	44	48-50	Pericentric inversion	RJ and Eastern SP to Southern Serra da Mantiqueira	Costa et al. 2011
		Rhipidomys leucodactylus (Tschudi, 1845)	-	44	46, 48, 52	Pericentric inversion	Northwestern Brazil (AM, AC, MT, RO, RR, AP and PA)	Zanchin et al. 1992, Silva and Yonenaga-Yassuda 1999, Patton et al. 2000, Bonvicino et al. 2008, Tribe 2015
		Rhipidomys macconnelli de Winton, 1900	-	44*	50*	Karyotype of specimens from Venezuela	AM (Serra da Neblina) and Western RR, above 1.000m of altitude	Aguilera et al. 1994, Bonvicino et al. 2008
		Rhipidomys macrurus (P. Gervais, 1855)	-	44	48-52	Pericentric inversion	Cerrado and Caatinga biomes, from CE to MT, and MG	Zanchin et al. 1992, Silva and Yonenaga-Yassuda 1999, Musser and Carleton 2005, Carvalho et al. 2012
		Rhipidomys mastacalis (Lund, 1840)	-	44	70, 74, 76, 80	Pericentric inversion	Atlantic Forest region, from PE to PR	Zanchin et al. 1992, Andrades-Miranda et al. 2002b, Paresque et al. 2004, Musser and Carleton 2005, Sousa 2005, Bonvicino et al. 2008, Carvalho et al. 2012, Tribe 2015
		Rhipidomys nitela Thomas, 1901	*Rhipidomys* sp. B	48; 50	68; 71, 72	Pericentric inversion in pair 8, addition and deletion of constitutive hetechromatin	Northcentral Brazil (AM, MT, AP, RR, PA, TO and GO)	Silva and Yonenaga-Yassuda 1999, Andrades-Miranda et al. 2002b, Tribe 2015
		Rhipidomys tribei B. M. de A. Costa, Geise, Pereira and L. P. Costa, 2011	-	44	50	-	Serra do Caraça, Southern MG	Zanchin et al. 1992, Costa et al. 2011
		Rhipidomys wetzeli A. L. Gardner, 1990	-	N/A	N/A	-	Northern Brazil	Fonseca et al. 1996, Tribe 2015
	Tribe Wiedomyini	*Wiedomys cerradensis* P. R. Gonçalves, Almeida & Bonvicino, 2005	-	60	88	-	Only known from its type locality (Southwestern BA)	Gonçalves et al. 2005
		Wiedomys pyrrhorhinos (Wied-Neuwied, 1821)	-	62	86, 90, 104	Pericentric inversion in the smallest pairs	Southern CE, Southeastern PI, and Western PB, PE, AL, BA and Northern MG	Maia and Langguth 1987, Gonçalves et al. 2005, Bonvicino et al. 2008, Souza et al. 2011

		Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Cricetidae - Subfamily Sigmodontinae	***Incertae sedis***	*Abrawayaomys ruschii* F. Cunha & Cruz, 1979	-	58	N/A	-	ES, RJ, SP, MG and SC	Bonvicino et al. 2008, Pereira et al. 2008
		Delomys altimontanus Gonçalves & Oliveira, 2014	-	82	86	-	Disjunction distribution in Itatiaia (RJ) and Caparaó (MG)	Gonçalves and Oliveira 2014
		Delomys dorsalis (Hensel, 1872)	*Thomasomys dorsalis collinus*, *D. collinus*	82	80	-	Atlantic Forest of Southeastern Brazil, from MG and ES to RS	Musser and Carleton 2005, Gonçalves and Oliveira 2014
		Delomys sublineatus (Thomas, 1903)	-	72	90	-	Atlantic Forest of Southeastern Brazil, from MG and ES to SC	Musser and Carleton 2005, Gonçalves and Oliveira 2014
		Juliomys ossitenuis L. P. Costa, Pavan, Leite, and Fagundes, 2007	-	20	36	-	Southern ES, and Eastern SP and MG	Costa et al. 2007, Bonvicino et al. 2008
		Juliomys pictipes (Osgood, 1933)	*Wilfredomys pictipes*	36	34	-	Southeastern Brazil, from MG to RS	Bonvicino and Otazu 1999, Musser and Carleton 2005
		Juliomys rimofrons J. A. Oliveira & Bonvicino, 2002	-	20	34	-	High altitudes at Serra da Mantiqueira, in SP, RJ and MG	Oliveira and Bonvicino 2002, Bonvicino et al. 2008
		Juliomys sp.	-	32	48	-	Aparados da Serra National Park, ES	Paresque et al. 2009
		Phaenomys ferrugineus (Thomas, 1917)	-	78	114	-	Restricted areas from Serra do Mar, in RJ and SP	Bonvicino et al. 2001b, Musser and Carleton 2005
		Wilfredomys oenax (Thomas, 1928)	-	N/A	N/A	-	Southern Brazil and Southeastern SP	Bonvicino et al. 2008
Family Ctenomyidae		*Ctenomys bicolor* Miranda-Ribeiro, 1914	-	40	64	-	RO	Stolz 2012
		Ctenomys flamarioni Travi, 1981	-	48	50-78	Variation in the amount of constitutive heterochromatin	Eastern RS	Massarini and Freitas 2005, Bonvicino et al. 2008
		Ctenomys ibicuiensis T. R. O. Freitas, Fernandes, Fornel & Roratto, 2012	-	50	68	-	Western RS	Bidau 2015
		Ctenomys lami T. R. O. Freitas, 2001	-	54-58	74-82; 84	Centric fusion/ fission in pairs 1 and 2; pericentric inversion	RS (Coxilha das Lombas, Northeastern Guaiba River to Southwestern Banks of Barros Lake)	Woods and Kilpatrick 2005, Freitas 2007
		Ctenomys minutus Nehring, 1887	-	42, 43, 44; 45; 46-51; 49-51; 48-51; 51; 52	74; 75/76; 77; 78; 78, 80; 79	Robertsonian rearrengements and tandem fusions	Eastern RS and SC	Freitas 1997, Gava and Freitas 2002, Freygang et al. 2004, Bonvicino et al. 2008
		Ctenomys nattereri Wagner, 1848	*Ctenomys boliviensis*	36	64	-	Southwestern MT and Southeastern RO	Anderson et al. 1987, Bonvicino et al. 2008, Stolz 2012
		Ctenomys rondoni Miranda-Ribeira, 1914	-	N/A	N/A	-	MT and RO	Bidau 2015

	Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family CtenomyidaE	*Ctenomys torquatus* Lichtenstein, 1830	-	40, 42, 44, 46	72	Robertsonian fusion; Variation in the amount of constitutive heterochromatin; secondary constricton	Southeastern RS	Freitas and Lessa 1984, Bonvicino et al. 2008, Fernandes et al. 2009
Family Cuniculidae	*Cuniculus paca* (Linnaeus, 1766)	-	74	98	-	All Brazilian States	Giannoni et al. 1991, Bonvicino et al. 2008
Family Dasyproctidae	*Dasyprocta azarae* Lichtenstein, 1823	*Dasyprocta aurea*	64	122	-	Southcentral Brazil, MG and SP	Souza et al. 2007, Bonvicino et al. 2008
	Dasyprocta croconota Wagler, 1831	-	N/A	N/A	-	Northeastern PA, Northwestern CE and Northermost TO	Bonvicino et al. 2008, Patton and Emmons 2015
	Dasyprocta fuliginosa Wagler, 1832	-	64; 65	116; 122	B chromosome	AM, AC, RO and Northwestern MT	Lima and Langguth 1998, Ramos et al. 2003, Bonvicino et al. 2008
	Dasyprocta iacki Feijó & Langguth, 2013	*Dasyprocta aguti*	64	122	-	Littoral zone in PB and PE	Lima and Langguth 1998, Feijó and Langguth 2013, Patton and Emmons 2015
	Dasyprocta leporina Linnaeus, 1758	-	64, 65	122-124	B chromosome	Northermost Brazil (AM, RR, AP and PA)	Ramos et al. 2003, Bonvicino et al. 2008, Patton and Emmons 2015
	Dasyprocta prymnolopha Wagler, 1831	*Dasyprocta nigriclunis*	64, 65	122	B chromosome	Northeastern Brazil, and Northern MG	Ramos et al. 2003, Woods and Kilpatrick 2005, Bonvicino et al. 2008
	Dasyprocta punctata Gray, 1842	-	N/A	N/A	-	Southeastern Brazil	Woods and Kilpatrick 2005, Patton and Emmons 2015
	Dasyprocta variegata Tschudi, 1845	-	64*	124	-	Western Brazil	Patton and Emmons 2015
	Dasyprocta sp.	-	64, 65	124	B chromosome	unknown distribution	Ramos et al. 2003
	Myoprocta acouchy (Erxleben, 1777)	-	62	118	-	RR, and Northeastern AM and PA	Hsu and Benirschke 1968, Bonvicino et al. 2008, Patton and Emmons 2015
	Myoprocta pratti Pocock, 1913	-	N/A	N/A	-	AC and Western AM	Bonvicino et al. 2008, Patton and Emmons 2015
Family Dinomyidae	*Dinomys branickii* Peters, 1873	-	64	98	-	AC and Southwesternmost AM	Bonvicino et al. 2008, Vargas and Ortiz 2010
Family Echimyidae	*Callistomys pictus* (Pictet, 1843)	-	42	76	-	Southeastern BA	Bonvicino et al. 2008, Ventura et al. 2008, Emmons and Leite 2015
	Carterodon sulcidens (Lund, 1838)	-	66	N/A	Secondary constriction in the forth largest pair	DF, GO, MT and MG	Carmignotto 2005, Bezerra and Bonvicino 2015, Present study

	Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Echimyidae	*Clyomys laticeps* (Thomas, 1909)	*Clyomys bishopi*	34; 32	58, 60, 62; 54	Pericentric inversion; Robertsonian rearrangement; secondary constriction in pair 1; addition of constitutive heterochromatin	MT, MS, GO, DF, SP and MG	Souza and Yonenaga-Yassuda 1984, Svartman 1989, Bonvicino et al. 2008, Bezerra et al. 2012
	Dactylomys boliviensis Anthony, 1920	-	118	168	-	AC	Dunnum et al. 2001, Woods and Kilpatrick 2005
	Dactylomys dactylinus (Desmarest, 1817)	-	94	144	-	AM, PA, RR, TO and Northern GO	Aniskin 1993, Bonvicino et al. 2008
	Echimys chrysurus (Zimmermann, 1780)	-	N/A	N/A	-	Southern AP, Northeastern PA and Northwestern MA	Bonvicino et al. 2008
	Echimys vieirai Iack-Ximenes, de Vivo & Percequillo, 2005	-	N/A	N/A	-	Central-Easternmost AM and Central-Westernmost PA	Bonvicino et al. 2008
	Euryzygomatomys spinosus (G. Fischer, 1814)	-	46	82	-	Eastern MG, SP and RJ, PR and Northern RS	Yonenaga 1975, Bonvicino and Bezerra 2015
	Isothrix bistriata Wagner, 1845	-	60	116	-	Northern AC and RO, Northeastern MT, and Southern AM	Leal-Mesquita 1991, Bonvicino et al. 2008
	Isothrix negrensis Thomas, 1920	-	60	112	-	Northern AM	Bonvicino et al. 2003b, Bonvicino et al. 2008
	Isothrix pagurus Wagner, 1845	-	22	38	-	Northeastern AM	Patton and Emmons 1985, Bonvicino et al. 2008
	Kannabateomys amblyonyx (Wagner, 1845)	-	98	126	-	Eastern Brazil, from ES to RS	Paresque et al. 2004, Bonvicino et al. 2008
	Lonchothrix emiliae Thomas, 1920	-	N/A	N/A	-	Eastern AM	Bonvicino et al. 2008
	Makalata didelphoides (Desmarest, 1817)	-	66	106	Secondary constriction in pair 11	AP, RR, Eastern AM, Western PA and TO, and Northern MT	Lima et al. 1998, Bonvicino et al. 2008
	Makalata macrura (Wagner, 1842)	-	N/A	N/A	-	AM and AC	Bonvicino et al. 2008
	Makalata obscura (Wagner, 1840)	-	N/A	N/A	-	Eastern PA and Westernmost MA	Bonvicino et al. 2008
	Mesomys hispidus (Desmarest, 1817)	-	60	116	-	Northern Brazil, and Northwestern MT	Leal-Mesquita 1991, Bonvicino et al. 2008
	Mesomys occultus Patton, da Silva & Malcolm, 2000	-	42	54	Secondary constriction in the smallest biarmed pair	Central AM	Patton et al. 2000, Woods and Kilpatrick 2005
	Mesomys stimulax Thomas, 1911	-	60	116	-	Eastern PA	Patton et al. 2000, Bonvicino et al. 2008
	Myocastor coypus (G. I. Molina, 1782)	-	42	76	-	RS	González and Brum-Zorilla 1995, Bonvicino et al. 2008, Fabre et al. 2016

	Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Echimyidae	*Phyllomys blainvillii* (Jourdan, 1837)	-	50	88, 94-96	Pericentric inversion	BA, SE, AL and PE, Southern CE, and Northern MG	Souza 1981, Leite 2003, Bonvicino et al. 2008, Machado 2010
	Phyllomys brasiliensis Lund, 1840	-	N/A	N/A	-	Central MG	Bonvicino et al. 2008
	Phyllomys dasythrix Hensel, 1872	-	72	108	-	Southern PR to RS	Leite 2003, Woods and Kilpatrick 2005, Machado 2010
	Phyllomys kerri (Moojen, 1950)	-	N/A	N/A	-	Ubatuba (SP)	Woods and Kilpatrick 2005
	Phyllomys lamarum (Thomas, 1916)	-	56	102	-	Eastern Brazil, from PB to MG	Woods and Kilpatrick 2005, Araújo et al. 2014
	Phyllomys lundi Y. L. R. Leite, 2003	-	N/A	N/A	-	Southern MG to RJ	Bonvicino et al. 2008
	Phyllomys mantiqueirensis Y. L. R. Leite, 2003	-	N/A	N/A	-	Serra da Mantiqueira (MG)	Bonvicino et al. 2008
	Phyllomys medius (Thomas, 1909)	-	96	108	-	From RJ to RS	Sbalqueiro et al. 1989, Bonvicino et al. 2008
	Phyllomys nigrispinus (Wagner, 1842)	-	84, 85	N/A	Secondary constriction in one acrocentric pair	Coast from RJ to PR, extending to inland Western SP	Leite 2003, Woods and Kilpatrick 2005, Delciellos et al. 2017
	Phyllomys pattoni Emmons, Leite, Kock & Costa, 2002	-	72; 76; 80	114; 148; 100, 108, 112	Pericentric inversion; centric fusion/ fission	From PB to Northeastern SP	Zanchin 1988, Leite 2003, Paresque et al. 2004, Woods and Kilpatrick 2005, Leite and Loss 2015
	Phyllomys sulinus Y. L. R. Leite, Christoff & Fagundes, 2008	-	92	102	-	Southern Brazil, from SP to RS	Yonenaga 1975, Leite 2003, Leite and Loss 2015
	Phyllomys thomasi (Ihering, 1897)	-	N/A	N/A	-	Ilha de São Sebastião (SP)	Woods and Kilpatrick 2005, Leite and Loss 2015
	Phyllomys unicolor (Wagner, 1842)	-	N/A	N/A	-	Southernmost BA	Bonvicino et al. 2008, Leite and Loss 2015
	Proechimys brevicauda (Günther, 1876)	-	28	48-50	Variations in FN due to difficulty in classifying the morphology of the small pairs	AC and Southern AM	Patton et al. 2000, Bonvicino et al. 2008
	Proechimys cuvieri Petter, 1978	-	28	46-48	Differences in the number of subtelocentrics and acrocentrics	Northern Brazil	Maia and Langguth 1993, Patton et al. 2000, Bonvicino et al. 2008
	Proechimys echinotrix M. N. F. da Silva, 1998	-	32	60	-	Northwestern AM	da Silva 1998, Bonvicino et al. 2008
	Proechimys gardneri M. N. F. da Silva, 1998	-	40	54, 56	Pericentric inversion; secondary constriction in the smallest submetacentric pair	Southern AM	da Silva 1998, Bonvicino et al. 2008, Eler et al. 2012
	Proechimys goeldii Thomas, 1905	-	24	44	-	Easternmost AM and Northwestern PA	Machado et al. 2005, Patton and Leite 2015
	Proechimys gr. *goeldii*	-	15	16	-	MT	Machado et al. 2005

	Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Echimyidae	*Proechimys guyannensis* (I. Geoffrey St.-Hilaire, 1803)	-	38, 44	52	One name with different karyotypes associated	Northeastern AM, Northern PA, Southeastern RR and AP	Machado et al. 2005, Bonvicino et al. 2008
	Proechimys hoplomyoides Tate, 1939	-	N/A	N/A	-	Northernmost RR	Bonvicino et al. 2008
	Proechimys kulinae M. N. F. da Silva, 1998	-	34	52	-	Southeastern AM	da Silva 1998, Patton et al. 2000, Bonvicino et al. 2008
	Proechimys longicaudatus (Rengger, 1830)	-	28	48-50	Pericentric inversion of pairs 3 and 11; addition/ deletion of constitutive heterochromatin	MT	Machado et al. 2005, Bonvicino et al. 2008
	Proechimys cf. *longicaudatus*	-	16, 17	14	Robertsonian rearrangement between X and the largest acrocentric chromosome; Multiple sex chromosome system (XX, XY1Y2)	MT	Amaral et al. 2013
	Proechimys pattoni M. N. F. da Silva, 1998	-	40	56	-	Western AC	Patton and Gardner 1972, da Silva 1998, Bonvicino et al. 2008
	Proechimys quadruplicatus Hershkovitz, 1948	-	28	42	-	Northcentral AM	Patton et al. 2000, Bonvicino et al. 2005b, Bonvicino et al. 2008
	Proechimys roberti Thomas, 1901	-	30	54-56	Pericentric inversion of pairs 13 and 14	Eastern PA, TO and GO, and Western MG and MA	Svartman 1989, Leal-Mesquita 1991, Machado et al. 2005, Ribeiro 2006, Bonvicino et al. 2008
	Proechimys simonsi Thomas, 1900	*Proechimys hendeei*	32	56-58	Pericentric inversion; secondary constriction in pair 8 of the karyotype with NF=56	AC and Southwestern AM	Patton and Gardner 1972, Gardner and Emmons 1984, Patton et al. 2000, Bonvicino et al. 2008
	Proechimys steerei Goldman, 1911	-	24	40-42	Pericentric inversion in pair 3 (smallest metacentric), with homo or heterozigous chromosomes	AC and Southwestern AM	Patton et al. 2000, Bonvicino et al. 2008
	Proechimys sp.	*Proechimys* gr. *longicaudatus*	30	52	-	Rio Jamari, RO	Leal-Mesquita 1991, Patton and Leite 2015
	Proechimys sp. A	*Proechimys* gr. *goeldii*	38	52	-	Rio Negro-Rio Aracá, AM	Bonvicino et al. 2005b
	Proechimys sp. B	-	46	50	-	RR and Northern AM	Bonvicino et al. 2005b, Bonvicino et al. 2008
	Thrichomys apereoides (Lund, 1839)	-	28	50, 52	Secondary constriction in pair 2	MG, Eastern GO and Western BA	Bonvicino et al. 2002a, Pessôa et al. 2004
	Thrichomys inermis (Pictet, 1843)	-	26	48	Secondary constriction in pair 2	BA and TO	Pessôa et al. 2004, Bonvicino et al. 2008
	Thrichomys laurentius Thomas, 1904	-	30	54	Secondary constriction in pair 1	Northeastern Brazil, except MA	Souza and Yonenaga-Yassuda 1982, Bonvicino et al. 2008

	Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Echimyidae	*Thrichomys aff. laurentius*	-	30	56	Secondary constriction in pair 1	Central Brazil	Bonvicino et al. 2002a, Braggio and Bonvicino 2004
	Thrichomys pachyurus Wagner, 1845	-	34	64	Secondary constriction in pair 2	Southern MT, and MS	Pessôa et al. 2004, Bonvicino et al. 2008
	Trinomys albispinus (I. Geoffrey St.-Hilaire, 1838)	-	60	116	Secondary constriction in pair 10	BA, SE and MG	Leal-Mesquita et al. 1993, Souza et al. 2006, Pessôa et al. 2015
	Trinomys dimidiatus (Günther, 1876)	-	60	116	Secondary constriction in pair 10	RJ and Northern SP	Pessôa et al. 2004, Bonvicino et al. 2008
	Trinomys eliasi (Pessôa & Reis, 1993)	-	38	112	Secondary constriction in pair 10	RJ	Pessôa et al. 2005, Bonvicino et al. 2008
	Trinomys gratiosus (Moojen, 1948)	*Trinomys* gr. *bonafidei*	56	108	Secondary constriction in pair 10	Southcentral ES to Southwestern RJ	Zanchin 1988, Woods and Kilpatrick 2005
	Trinomys iheringi (Thomas, 1911)	*Proechimys iheringi iheringi*	60-65	116	1 to 5 B chromosomes; secondary constriction in pair 7	Coast from Southern RJ to Northern PR	Yonenaga-Yassuda et al. 1985, Fagundes et al. 2004, Bonvicino et al. 2008
	Trinomys mirapitanga Lara, Patton and Hingst- Zaher, 2002	-	N/A	N/A	-	BA	Lara et al. 2002, Woods and Kilpatrick 2005
	Trinomys moojeni (Pessôa, Oliveira & Reis, 1992)	-	56	106	-	Only known from the type locality (MG)	Corrêa et al. 2005, Woods and Kilpatrick 2005
	Trinomys paratus (Moojen, 1948)	-	58	112	Secondary constriction in long arm of a median size autosome	South-central ES and easternmost MG	Bonvicino et al. 2008, Lazar et al. 2017
	Trinomys setosus (Desmarest, 1817)	*Trinomys s. setosus* and *Trinomys s. elegans*	56	108, 104	NFs refer to each subspecies, respectively	Eastern Brazil, from SE to *ES* and MG	Bonvicino et al. 2008, Pêssoa et al. 2015
	Trinomys yonenagae (P. L. B. Rocha, 1996)	-	54	104	Secondary constriction in pair 10	BA, left bank of Rio São Francisco	Leal-Mesquita et al. 1992, Bonvicino et al. 2008
	Toromys grandis (Wagner, 1845)	-	N/A	N/A	-	Eastern AM and PA	Bonvicino et al. 2008
Family Erethizontidae	*Chaetomys subspinosus* Olfers, 1818	-	52	76	-	ES and Southeastern BA	Bonvicino et al. 2008, Vilela et al. 2009
	Coendou insidiosus (Olfers, 1818)	*Sphiggurus insidiosus*	62	76	-	Eastern Brazil, from CE to ES	Lima 1994, Bonvicino et al. 2008, Voss 2015c
	Coendou melanurus (Wagner, 1842)	*Sphiggurus melanurus*	72	76	-	Northernmost Brazil (AM, RR, AP and PA)	Bonvicino et al. 2002b, Bonvicino et al. 2008, Voss 2015c
	Coendou nycthemera (Olfers, 1818)	-	N/A	N/A	-	Easternmost AM and PA	Bonvicino et al. 2008, Voss 2015c
	Coendou prehensilis (Linnaeus, 1758)	-	74	82	-	From Northern to Southeastern Brazil	Lima 1994, Bonvicino et al. 2008, Voss 2015c
	Coendou roosmalenorum Voss and da Silva, 2001	*Sphiggurus roosmalenorum*	N/A	N/A	-	Centraleastern AM	Bonvicino et al. 2008, Voss 2015c

	Species	Synonyms	2n	FN	Karyotypic Variations	Distribution	References
Family Echimyidae	*Coendou speratus* Mendes Pontes, Gadelha, Melo, de Sá, Loss, Caldara Junior, Costa & Leite, 2013	-	N/A	N/A	-	Eastern PE and AL	Mendes-Pontes et al. 2013, Voss 2015c
	Coendou spinosus (F. Cuvier 1823)	*Sphiggurus spinosus*, *S. villosus*	42	76	-	Southern Brazil, Southeastern MG, and Eastern SP and RJ	Mendes-Pontes et al. 2013, Voss 2015c
Family Muridae	*Mus musculus* Linnaeus, 1758	-	40	38	-	All Brazilian States	Bonvicino et al. 2008, present study
	Rattus rattus Linnaeus, 1758	-	38	58-59	Pericentric inversion in pair 8	All Brazilian States	Kasahara and Yonenaga-Yassuda 1981, Kasahara and Yonenaga-Yassuda 1984, Bonvicino et al. 2008
	Rattus norvegicus Berkenhout, 1769	-	42	64	-	All Brazilian States	Bianchi et al. 1969, Bonvicino et al. 2008
Family Sciuridae	*Guerlinguetus aestuans* (Linnaeus, 1766)	*Guerlinguetus gilvigularis*, *G. poaiae*	N/A	N/A	-	RR, AP, AM, PA and Central MT	Bonvicino et al. 2008, De Vivo and Carmignotto 2015
	Guerlinguetus brasiliensis (Gmelin, 1788)	*Guerlinguetus alphonsei*, *G. henseli*, *G. ingrami*	40	74, 76	Pericentric inversions	Disjunct distribution of Amazonian, Caatinga, and Coastal Brazil	Lima and Langguth 2002, Fagundes et al. 2003, De Vivo and Carmignotto 2015
	Hadrosciurus igniventris (Wagner, 1842)	*Sciurus igniventris*	N/A	N/A	-	Northern Brazil, Southern Amazon River	Bonvicino et al. 2008, De Vivo and Carmignotto 2015
	Hadrosciurus pyrrhinus (Thomas, 1898)	*Sciurus igniventris*, *S. pyrrhonotus*, *S. pyrrhinus*	N/A	N/A	-	Western Brazilian Amazonia	Patton et al. 2015
	Hadrosciurus spadiceus (Olfers, 1818)	*Sciurus spadiceus*	40	76	-	Central to Southern AM, AC, RO, and Western PA and MT	Lima and Langguth 2002, Bonvicino et al. 2008, De Vivo and Carmignotto 2015
	Microsciurus flaviventer (Gray, 1867)	-	N/A	N/A	-	Northern Amazon River, Brazil	Bonvicino et al. 2008
	Notosciurus pucheranii (Fitzinger, 1867)	*Guerlinguetus ignitus*	N/A	N/A	-	Northwestern MT, Western AC and Southwestern AM	Bonvicino et al. 2008
	Sciurillus pusillus (I. Geoffrey St.-Hilaire, 1803)	-	N/A	N/A	-	Eastern AM and Western PA	Bonvicino et al. 2008

Abbreviations: Brazilian states AC: Acre; AL: Alagoas; AP: Amapá; AM: Amazonas; BA: Bahia; CE: Ceará; DF: Distrito Federal; ES: Espírito Santo; GO: Goiás; MA: Maranhão; MG: Minas Gerais; MS: Mato Grosso do Sul; MT: Mato Grosso; PA: Pará; PB: Paraíba; PE: Pernambuco; PI: Piauí; PR: Paraná; RJ: Rio de Janeiro; RN: Rio Grande do Norte; RO: Rondônia; RR: Roraima; RS: Rio Grande do Sul; SC: Santa Catarina; SE: Sergipe; SP: São Paulo; TO: Tocantins. N/A means that information is not available and (*) means that data do not refer to Brazilian specimens.

Cytogenetic preparation

Chromosome preparations of *Carterodon sulcidens*, the five samples of *Mus musculus*, four *Neacomys a. amoenus*, and a specimen of *Neacomys* from Vila Rica, Mato Grosso State, were obtained *in vivo* from bone marrow and spleen, following Ford and Hamerton (1956) or *in vitro* from fibroblast culture (Freshney 1986). Conventional Giemsa staining was performed to determine the diploid and fundamental numbers, and C-banding and Ag-NOR were performed according to Sumner (1972) and Howell and Black (1980), respectively.

Molecular phylogeny analyses of *Neacomys*

DNA was extracted from the liver or muscle with Chelex 5% (Bio-Rad) (Walsh et al. 1991) of five specimens of *Neacomys*. DNA of the specimen from Vila Rica, Mato Grosso State, was extracted from fibroblast cell culture using DNeasy Blood and Tissue kit (Qiagen, catalog number 69506).

PCR was performed in a thermal cycler (Eppendorf Mastercycler ep Gradient, Model 5341) using primers MVZ05 (5-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3) and MVZ16 (5-AAA TAG GAA RTA TCA YTC TGG TTT RAT-3) (Irwin et al. 1991, Smith and Patton 1993, respectively). PCR mixture contained 30 ng of DNA, 25 pmol of each primer, 0.2 mM of dNTP, 2.52 µL of reaction buffer (50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl; pH 8.8) and 0.2 units of Taq DNA polymerase (Invitrogen). Thirty-nine amplification cycles were performed, consisting of denaturation at 94 °C for 30 s, annealing at 48 °C for 45 s, extension at 72 °C for 45 s and the final extension at 72 °C for 5 min. The PCR products were separated using 1% agarose gel in TAE buffer. Sequencing was conducted using BigDye (DNA "Big Dye Terminator Cycle Sequencing Standart," Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). All sequences were submitted to a comparative similarity search on BLAST (Basic Local Alignment Search Tool) before the alignment. Alignments were performed by using Muscle (Edgar, 2004) implemented in Geneious 4.8.5 (Biomatters). GenBank access numbers are provided in Suppl. material 1.

Models of nucleotide substitution were selected using Bayesian Information Criterion (BIC), implemented in PartitionFinder, version 1.1.1 (Lanfear et al. 2012). Approximately 673 bp were used to perform Maximum Likelihood (ML) in GARLI 2.0 (Bazinet et al. 2014) and Bayesian Inference (BI) in MrBayes 3.04b (Ronquist and Huelsenbeck 2003), using 69 additional *Neacomys* sequences downloaded from GenBank, plus sequences of *Euryoryzomys russatus* (Wagner, 1848), *Holochilus brasiliensis* (Desmarest, 1819) and *Oligoryzomys nigripes* (Olfers, 1818) as the outgroup (see Suppl. material 1).

Results

The current review encompasses all rodent species which up to the present have been reported in Brazil, comprising 271 species from 10 families (Musser and Carleton 2005, Patton et al. 2015, Fabre et al. 2016). Diploid number ranges from 2n = 9, 10 in *Akodon* sp. n. to 2n = 118 in *Dactylomys boliviensis* Anthony, 1920 (Table 1). It is noteworthy that 38 species (14%) lack any cytogenetic data. Besides, nine species present only the diploid number with no information about the fundamental number.

Many species show chromosome rearrangements leading to variation in diploid and fundamental numbers. Also, more than one diploid number was associated with one single species, suggesting that they could represent species' complexes. Additionally, new karyotypes were assigned to 22 species highlighting them as candidate species, which have not been formally described yet.

All comments below refer to the data compiled and presented in Table 1.

Family Caviidae

From a total of ten species, cytogenetic data is lacking for only one: *Galea flavidens* (Brandt, 1835). The diploid number varied from 2n = 52 in *Kerodon acrobata* Moojen, 1997 and *K. rupestris* (Wied-Neuwied, 1820) to 2n = 66 in *Hydrochoerus hydrochaeris* (Linnaeus, 1766). Currently, polymorphism of autosomal chromosomes has been described for *Cavia porcellus* (Linnaeus, 1758), pericentric inversions for *C. magna* Ximénez, 1980 and *K. rupestris*, and Robertsonian rearrangement for *C. magna* (Maia 1984, Gava et al. 2011) (Table 1).

Family Cricetidae

Subfamily Sigmodontinae

Tribe Akodontini

This is the second most diverse tribe in the subfamily Sigmodontinae. Only five out of 42 species (D'Elía and Pardiñas 2015) that occur in Brazil lack diploid number information (Table 1). However, for one species, *Akodon toba* Thomas, 1921, such information is available only for Paraguayan specimens. In addition to the species on which there is no information on the diploid number, four species of the genus *Oxymycterus* Waterhouse, 1837 have not had their fundamental number established, yet.

In this tribe, the diploid number varied from 2n = 9, 10 in *Akodon* sp. n. to 2n = 70 in *Bibimys labiosus* (Winge, 1887). B chromosomes are found in *Akodon montensis*

and *Blarinomys breviceps* (Winge, 1887). Also, pericentric inversions were described in three species of the tribe, Robertsonian rearrangements in six, and reciprocal translocation in one. These rearrangements are reported for *Akodon cursor* (although some authors consider *A. cursor* as a species complex, because of the molecular phylogeny – see Geise et al. 2001, Silva et al. 2006), *Akodon* sp. n., *Akodon montensis*, *Blarinomys breviceps*, *Brucepattersonius griserufescens* Hershkovitz, 1998, *Deltamys kempi* Thomas, 1917, *Necromys lasiurus* (Lund, 1840), *Scapteromys meridionalis* Quintela, Gonçalves, Althoff, Sbalqueiro, Oliveira & Freitas, 2014, and *Thalpomys lasiotis* Thomas, 1916.

Sex chromosome variation is also common, occurring in six species. It is also remarkable that *Deltamys kempi* is one of the few rodents to which multiple sex system has been described ($X_1X_1X_2X_2$/ X_1X_2Y) (Sbalqueiro et al. 1984).

Cytogenetic studies have proved to be a useful tool in the recognition of species, mainly in the case of the cryptic and sympatric species as *Akodon cursor* and *A. montensis*. On the other hand, karyotype was less variable in some other Akodontini genus (for instance *Brucepattersonius* and *Oxymycterus*), and in this case, they could not be distinguished cytogenetically. This reveals the need for gathering cytogenetic, molecular and morphological data in taxonomic studies.

Tribe Ichthyomyini

Two species of *Neusticomys*, *N. oyapocki* (Dubost & Petter, 1979) and *N. ferreirai* Percequillo, Carmignotto & Silva, 2005, occur in Brazil and karyotype information is available only for *N. ferreirai* (Table 1). Karyotype shows 2n = 92, FN = 98, and autosomes consist of four biarmed pairs and 41 acrocentrics. X chromosome is a large metacentric and Y is the largest acrocentric (Percequillo et al. 2005).

Tribe Oryzomyini

Comprising 73 species up to now, this tribe alone comprises about 47% of the Sigmodontinae diversity. Notwithstanding, it is one of the best cytogenetically studied taxa of Brazilian rodents, and cytogenetic information on fundamental number lacks for only one species: *Neacomys guianae* Thomas, 1905. In Brazilian representatives the diploid number varied from 2n = 34 in *Neacomys musseri* Patton, da Silva & Malcolm, 2000 to 2n = 86 in *Zygodontomys brevicauda* (J. A. Allen & Chapman, 1893).

Pericentric inversion (n = 13) and Robertsonian rearrangements (n = 8) are common rearrangements, as well as sex chromosomes variations, that were described in 12 species and correlated to addition/deletion of constitutive heterochromatin and pericentric inversions.

Besides, Oryzomyini is also the tribe with more species having supernumerary chromosomes (n = 6). Remarkably, B chromosomes in this tribe present different morphology and composition, not only between, but also within the same species. For

instance, *Nectomys squamipes* Brants, 1827 presents from one to three supernumeraries that could be large/medium submetacentric or medium acrocentric, with interstitial or entire long arm C-banded, with late or early replication and with or without interstitial telomeric sites (Silva and Yonenaga-Yassuda 1998b). Differences were also described in Bs of *Holochilus brasiliensis*, *Nectomys rattus* Pelzeln, 1883, and *Oligoryzomys flavescens* (Waterhouse, 1837) (Silva and Yonenaga-Yassuda 2004). Recently, FISH with *Holochilus brasiliensis* probes of sex chromosomes (X and Y) and both supernumeraries (B1 and B2) were performed, revealing positive signal on sex chromosome of 12 Oryzomyini species and Bs of *Holochilus brasiliensis*, *Nectomys rattus* and *N. squamipes* (Ventura et al. 2015). No signal was observed in Bs of *Oligoryzomys flavescens* and *Sooretamys angouya* (G. Fischer, 1814), though, corroborating that supernumeraries in this group may have had independent origins (Ventura et al. 2015).

Karyotype information proved to be important in this tribe, since many species present species-specific karyotypes. For example, species of the genus *Oligoryzomys* are morphologically very similar but they present different karyotypes: *O. mattogrossae* (J. A. Allen, 1916) (2n = 62, FN = 64), *O. microtis* (J. A. Allen, 1916) (2n = 64, FN = 64,66), *O. moojeni* Weksler & Bonvicino, 2005 (2n = 70, FN = 72, 74, 76), *O. nigripes* (2n = 62, FN = 80-82), *O. stramineus* Bonvicino & Weksler, 1998 (2n = 52, FN = 68-70), *O. utiaritensis* J. A. Allen, 1916 (2n = 72, FN = 76) (Almeida and Yonenaga-Yassuda 1991, Bonvicino and Weksler 1998, Andrades-Miranda et al. 2001a, Agrellos et al. 2012, Di-Nizo 2013).

Chromosome data also show evidence that distinctive karyotypes are being attributed to the same name, for instance *Euryoryzomys macconnelli* (Thomas, 1910), *E. lamia* (Thomas, 1901), *Hylaeamys yunganus* (Thomas, 1902), *Oecomys cleberi* Locks, 1981, *Oecomys paricola* (Thomas, 1904), *Oecomys roberti* (Thomas, 1904) and *Zygodontomys brevicauda* (Andrades-Miranda et al. 2000, Patton et al. 2000, Suárez-Villota et al. 2017).

Additionally, some species could not be identified by chromosome data alone, because they share the same karyotype. This is the case of *Cerradomys marinhus* (Bonvicino, 2003) and *Pseudoryzomys simplex* (Winge, 1887) (2n = 56, FN = 54 - except for the morphology of the Y); *Euryoryzomys emmonsae* (Musser et al., 1998), *E. russatus* and *E. nitidus* (Thomas, 1884) (2n = 80, FN = 86); *Hylaeamys laticeps* (Lund, 1840) and *H. seuanezi* (Weksler et al., 1999) (2n = 48, FN = 60); *H. oniscus* (Thomas, 1904) and *H. perenensis* (J. A. Allen, 1901) (2n = 52, FN = 62); *Neacomys dubosti* Voss et al., 2001 and *N. amoenus* (2n = 64, FN = 68); *Oecomys bahiensis* (Hershkovitz, 1960), *Oecomys catherinae*, and *Oecomys concolor* (Wagner, 1845), *Oecomys* sp. 2 and sp. 3 (2n = 60, FN = 62); *Drymoreomys albimaculatus* Percequillo, Weksler & Costa, 2011 and *Oecomys* sp. 4 (2n = 62, FN = 62 - although ITS was observed in *Drymoreomys* but not in *Oecomys* – see Suárez-Villota et al. 2013 and Malcher et al. 2017); and *Holochilus brasiliensis* and *Nectomys squamipes* (standard karyotypes: 2n = 56, FN = 56). Also, although not distributed in Brazil, *Oligoryzomys brendae* Massoia, 1998 is found sympatric to *O. chacoensis* (Myers & Carleton, 1981) in Argentina and both possess 2n = 58, FN = 74.

Just as in all hierarchical levels of rodents' taxonomy, cytogenetic diversity is underestimated in this tribe. For instance, recently, Silva et al. (2015) described two new

cytotypes for *Neacomys*: 2n = 58, FN = 64, from samples collected in Marabá, and 2n = 58, FN = 70, from samples collected in Chaves, Marajó Island, localities from Pará State. According to the authors, both cytotypes differed in the number of biarmed pairs due to amplification/deletion of constitutive heterochromatin in the short arms of pairs 24, 26, and 27 (from Marajó Island) and pericentric inversion involving pairs 28 (metacentric) and 24 (acrocentric) from Marajó Island and Marabá, respectively. These karyotypes could not be assigned to any species described so far, and molecular phylogeny of these samples corroborates the cytogenetic data that it might be a new species (Silva et al. 2015).

Herein, we describe the same diploid (2n = 58), but with a different fundamental number (66) to *Neacomys* collected in Vila Rica, Mato Grosso State (approximately 700 km from those samples described by Silva et al. 2015). The karyotype comprises 23 acrocentric pairs decreasing in size (pair 1 is the largest of the complement), and five small biarmed pairs. The X chromosome is a large submetacentric, and the Y is a small submetacentric (Fig. 1a). The C-banding pattern shows constitutive heterochromatin at the pericentromeric regions of all autosomes, and in the short arm of both X and Y (Fig. 1b).

For phylogenetic analyses, the best model selected for the mitochondrial gene (cyt-*b*) was GTR+I+G. Our molecular phylogeny suggests that this specimen with 2n = 58, FN = 66, from Vila Rica may be an undescribed species that belongs to the same one reported by Silva et al. (2015) with 2n = 58, FN = 64, but with a new fundamental number, probably due to pericentric inversions (Fig. 2). Two structured clades of *Neacomys* with 2n = 58 were recovered: one with samples with FN = 70, and the other with FN = 64 and 66. Additionally, a sample from Igarapé-Açu (MTR12842), Rio Abacaxis (Amazonas, Brazil) was recovered as the sister group of these two clades. Although the phylogenetic reconstruction lacks *N. tenuipes* Thomas, 1900 (because the unique sequence available in GenBank has only 177pb), it is unlikely that samples with 2n = 58 belong to *N. tenuipes* once this species is distributed in Colombia and Venezuela and did not nest in the clade of *N. tenuipes* of the molecular phylogeny presented by Silva et al. (2015). In addition, our phylogenetic reconstruction recovered *Neacomys* as monophyletic with high support values (1PP/ 99ML). ML and IB analyses recovered the same topology.

Tribe Phyllotini

In Brazil, this tribe was initially composed only of the genus *Calomys* Waterhouse, 1837. However, due to sampling efforts, a new genus was recently added, *Calassomys* Pardiñas, Lessa, Salazar-Bravo & Câmara, 2014. The diploid number varied from 2n = 36 in *Calomys cerqueirai* to 2n = 66 in *Calomys tener* and *Calomys expulsus*, although the latter presents two different diploid numbers and karyotypes associated to its name, therefore highlighting the need for further investigation (Bonvicino and Almeida 2000, Mattevi et al. 2005). Cytogenetic information is available for all the representatives,

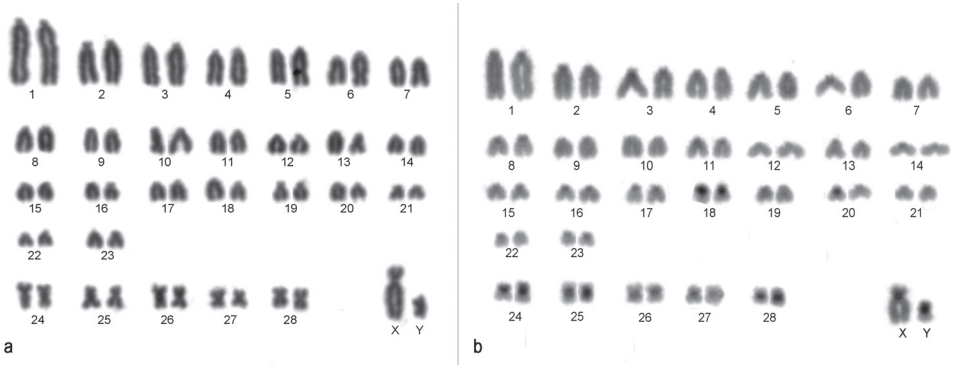

Figure 1. Karyotype of a male of *Neacomys* 2n=58, FN=66, from Vila Rica, Mato Grosso State, Brazil. **a** Giemsa-staining **b** C-banding.

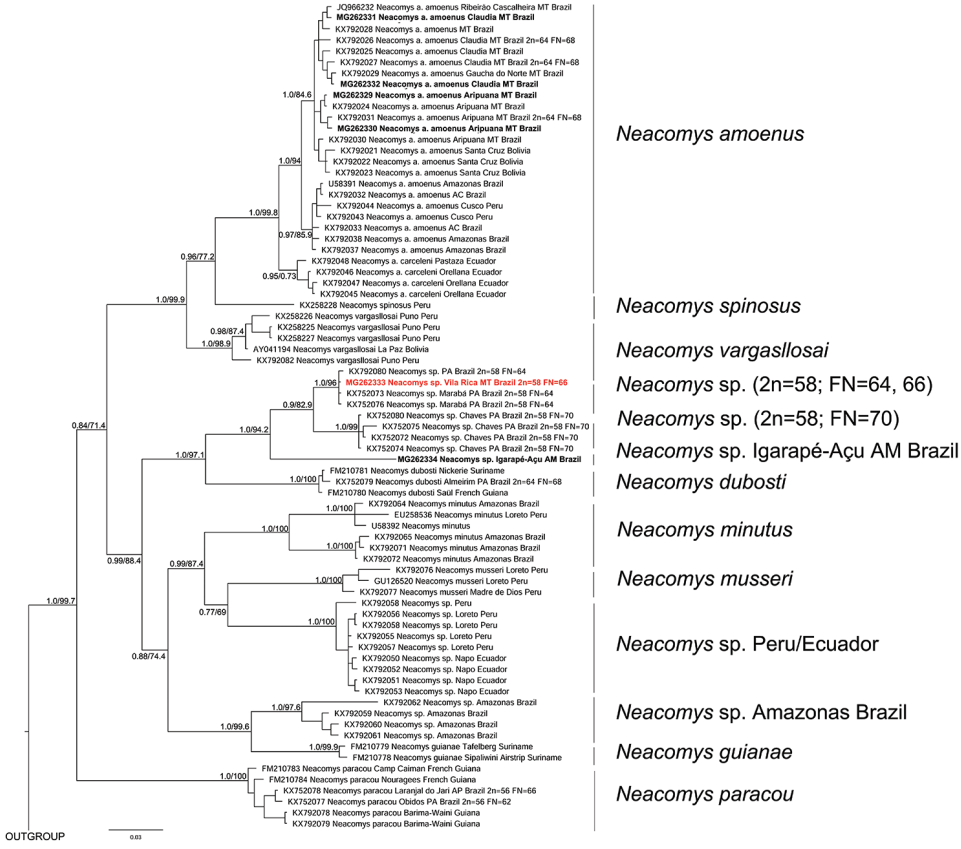

Figure 2. Bayesian phylogenetic hypothesis of *Neacomys* based on cyt-*b*. Numbers in the nodes indicate BI posterior probability (PP) and bootstrap support (ML), respectively. Individual from Vila Rica, Mato Grosso State with 2n=58, FN=66, is highlighted in red and the other samples analysed in this work are in bold.

and it is an important tool for the recognition of species (cytotaxonomy). One species presents centric fusion (*Calomys cerqueirai*) (Colombi and Fagundes 2014).

Tribe Reithrodontini

In Brazil, the only representative of this tribe is *Reithrodon typicus* Waterhouse, 1837. This species possesses a low diploid number (2n = 28) and occurs on the border of Uruguay (Freitas et al. 1983, Pardiñas et al. 2015c) (Table 1).

Tribe Sigmodontini

Only one species of this tribe can be found in Brazil, *Sigmodon alstoni* (Thomas, 1881). Voss (1992) karyotyped 11 specimens from three localities at Venezuela with 2n = 78, 80 and 82, but the picture of the karyotypes and the fundamental numbers were not reported. Also, the author suggested that Robertsonian rearrangement is a plausible explanation for the variation observed. There have been no Brazilian representatives of this species karyotyped so far.

Tribe Thomasomyini

This tribe is represented by only two genera in Brazil: *Rhipidomys* Tschudi, 1845 and *Rhagomys* Thomas, 1886. The diploid number varied from 2n = 36 in *Rhagomys rufescens* (Thomas, 1886) to 2n = 50 in *Rhipidomys nitela* Thomas, 1901. Apart from *R. nitela*, which possesses 2n = 48 (samples from Roraima State) or 50 (samples from Manaus, Amazonia State), in general, the karyotype is not informative for *Rhipidomys*, since nine species present the same diploid number (2n = 44), and two species lack karyotype data (Silva and Yonenaga-Yassuda 1999, Tribe 2005). In fact, Tribe (2015) provisionally inserted the 2n = 50 samples in *R. nitela* but reiterated that they need taxonomic revision. Pericentric inversion, found in six species, plays an important role in the genus, and this is reflected in the variation of the fundamental number. Two species lack cytogenetic data: *Rhipidomys ipukensis* R. G. Rocha, Costa & Costa, 2011 and *R. wetzeli* A. L. Gardner, 1990.

Tribe Wiedomyini

This tribe is composed of two species: *Wiedomys pyrrhorhinos* (Wied- Neuwied, 1821) and *W. cerradensis* P. R. Gonçalves, Almeida & Bonvicino, 2005. Both occur in Brazil with disjunctive distribution (*W. pyrrhorhinos* at Caatinga, and *W. cerradensis* at Cerrado) and possess different karyotypes (2n = 62 and 60, respectively) (Maia and

Langguth 1987, Gonçalves et al. 2005). Recent molecular studies indicate that *W. pyrrhorhinos*, may represent a species complex with Rio São Francisco acting as a barrier to the populations from both river banks (Di-Nizo *in prep*.). Pericentric inversions have also been described for this species.

Incertae sedis

This group comprises the genera *Abrawayaomys* F. Cunha & Cruz, 1979, *Delomys* Thomas, 1917, *Juliomys* E. M. González, 2000, *Phaenomys* Thomas, 1917, and *Wilfredomys* Avila-Pires, 1960, which could not be inserted into any other tribes, according to phylogenetic and morphological analyses (Musser and Carleton 2005, Patton et al. 2015). Cytogenetic information is available for all species, except one, *Wilfredomys oenax* (Thomas, 1928), and is helpful for distinguishing species of the genus *Delomys* and *Juliomys*.

Family Ctenomyidae

This family comprises a single genus, *Ctenomys*, which presents a great variation in diploid numbers, especially *C. lami* T. R. O. Freitas, 2001, *C. minutus* Nehring, 1887 and *C. torquatus* Lichtenstein, 1830 for which Robertsonian rearrangements and *in tandem* fusions were described (Freitas and Lessa 1984, Fernandes et al. 2009). The diploid number varied from 36 in *Ctenomys nattereri* Wagner, 1848 to 58 in *C. lami*. Only one species out of eight lacks karyotype information. Cytogenetic data was useful for recognizing *Ctenomys bicolor* Miranda- Ribeiro, 1914, *C. ibicuiensis* T. R. O. Freitas, Fernandes, Fornel & Roratto, 2012 and *C. nattereri*, because it presents exclusive karyotype (Stoulz 2012). Pericentric inversion has been described for *C. lami* and *in tandem* fusions for *C. minutus*.

Family Cuniculidae

This family is represented by a single species, *Cuniculus paca* (Linnaeus, 1766), with a wide distribution and unique karyotype (2n = 74, FN = 98) (Giannoni et al. 1991, Bonvicino et al. 2008).

Family Dasyproctidae

This family comprises two genera: *Dasyprocta* Illiger, 1811, with nine species, and *Myoprocta* Thomas, 1903, with two species (Patton and Emmons 2015). There is no cytogenetic data known for three species (Table 1). The diploid number in the Family varied from 62 to 65, and in the genus *Dasyprocta*, from 64 to 65, due to the presence of B chromosomes in four species (Ramos et al. 2003).

Family Dinomyidae

This family possesses only one species, *Dinomys branickii* Peters, 1873, to which the karyotype is 2n = 64, FN = 98 (Table 1).

Family Echimyidae

Even being the second largest Brazilian rodent family, a remarkable gap regarding cytogenetic data of this family still remains, with 14 species out of 68 lacking such information. This represents about 37% of all the unknown karyotypic information of all Brazilian rodents.

Diploid numbers varied from 2n = 15 in *Proechimys goeldii* Thomas, 1905 to 118 in *Dactylomys boliviensis*. B chromosomes have been described for one species: *Trinomys iheringi* (Thomas, 1911) (Yonenaga-Yassuda et al. 1985), pericentric inversion for seven species, and Robertsonian rearrangement for three. A multiple sex chromosome system was described for *Proechimys* cf. *longicaudatus* (Amaral et al. 2013), and addition/deletion of constitutive heterochromatin was described for *Clyomys laticeps* (Thomas, 1909) and *P. longicaudatus* (Rengger, 1830) (Souza and Yonenaga-Yassuda 1984, Bezerra et al. 2012, Machado et al. 2005). Secondary constriction is a characteristic feature of several species, occurring in *Carterodon sulcidens* (this work), *Clyomys laticeps*, *Mesomys occultus* Patton, da Silva & Malcolm, 2000, *Makalata didelphoides* (Desmarest, 1817), *Proechimys gardneri* M. N. F. da Silva, 1998, all five *Thrichomys* E.- L. Trouessart, 1880 species, and seven species of *Trinomys* Thomas, 1921.

Within this family, there are also cases in which different diploid numbers are assigned to the same name. In the case of *Clyomys laticeps*, the 2n = 34, FN = 58, 60, 62 and 2n = 32, FN = 54, the karyotypes are very similar, and differ by a Robertsonian rearrangement and pericentric inversion (2n = 32). Also, species such as *Phyllomys pattoni* Emmons, Leite, Kock & Costa, 2002 and *Proechimys guyannensis* E. Geoffroy, 1803 should be investigated by molecular phylogeny and morphology, because they are prone to either represent species-complex or have taxonomic misidentification.

In this work, the karyotype of *Carterodon sulcidens* is being described for the first time, showing 2n = 66. Since the animal was a female, it was not possible to recognize the X chromosomes and the exact morphology of the small pair, so we could not establish the fundamental number. Karyotype is composed of 32 acrocentric pairs decreasing in size and presumably one biarmed pair (pair 33). Also, the fourth largest pair possesses a remarkable secondary constriction (Fig. 3a). Constitutive heterochromatin is located in the pericentromeric region of all autosomes (Fig. 3b). Ag-NOR showed signals in the secondary constriction of pair 4 (Fig. 3b inset).

Within the Echimyidae Family, the only other species with 2n = 66 described so far is *Makalata didelphoides*, but its karyotype presents 20 pairs of metacentric chromosomes, which clearly differs from the karyotype of *Carterodon sulcidens*.

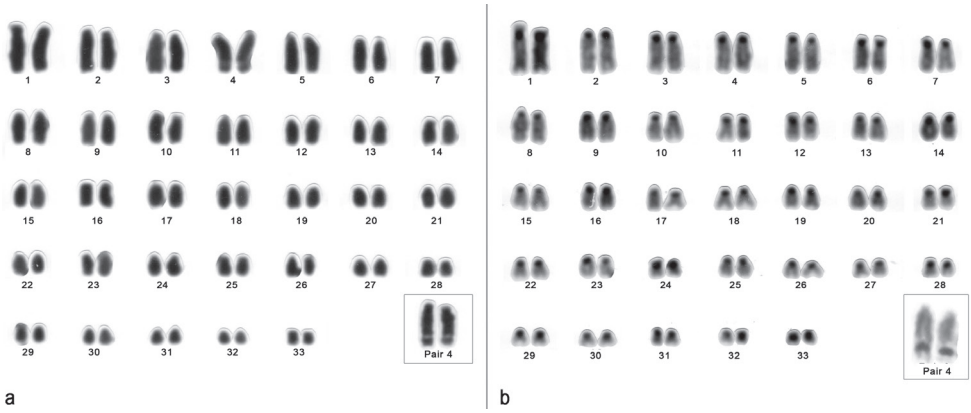

Figure 3. Karyotype of a female of *Carterodon sulcidens* with 2n=66 from Serra da Mesa, Goiás State, Brazil. **a** Giemsa-staining. Inset: Pair 4 with evident secondary constriction **b** C-banding. Inset: Pair 4 after silver nitrate staining.

Family Erethizontidae

Three out of eight species lack cytogenetic information. The diploid number varied from 42 in *Coendou spinosus* (F. Cuvier, 1823) to 74 in *C. prehensilis* (Linnaeus, 1758) (Lima 1994, Mendes-Pontes et al. 2013) (Table 1).

Family Muridae

This family (represented by the genera *Mus* and *Rattus*) was introduced from Europe, and even though it is not a native, it is currently widespread throughout Brazil (Musser and Carleton 2005).

Little is known about the cytogenetics of the *Mus musculus* Brazilian populations because this species seems to be negglected. The present paper features the first picture of *Mus musculus* karyotype from Brazil. This species presented 2n = 40, FN = 38, with all chromosomes acrocentrics. C-banding was restricted to the centromeric region of all chromosomes (Fig. 4). Sex chromosomes could only be recognized after G-banding (not showed) because they have similar morphology compared to the autosomes.

For the black rat *Rattus rattus* Linnaeus, 1758, diploid number of South America population is the same as those from Oceania (2n = 38), and Kasahara and Yonenaga-Yassuda (1981) described pericentric inversion for individuals from São Paulo, Brazil.

Family Sciuridae

Cytogenetic data is unknown for almost the entire family. For the two species to which chromosome information is known, diploid number is 2n = 40, and pericentric inver-

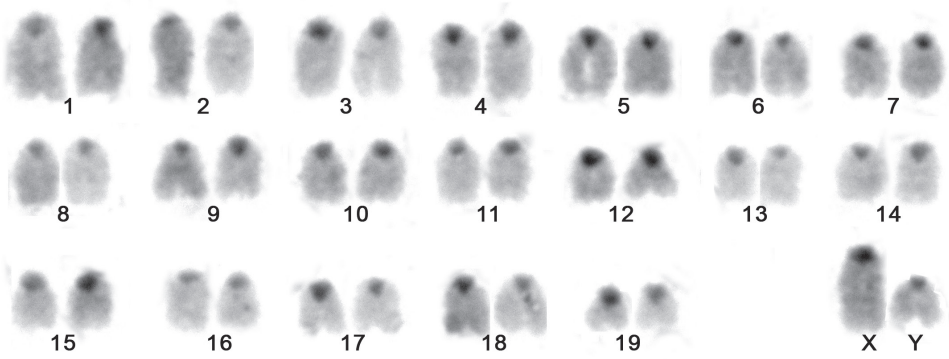

Figure 4. Karyotype after C-banding of a male of *Mus musculus* with 2n=40, FN=38, from Guará, São Paulo State, Brazil.

sion has been described for one of them, *Guerlinguetus brasiliensis* (Gmelin, 1788) (Lima and Langguth 2002, Fagundes et al. 2003) (Table 1).

Discussion

Advances since the last revision

The last cytogenetic revision on Brazilian rodents, published in 1984, described the karyotype of 62 species, mainly from South and Southeast Brazil (Kasahara and Yonenaga-Yassuda 1984). This paper compiles the karyotype of 271 species distributed throughout Brazil, representing an increase of more than 300%.

Since then, new cytotypes have been attributed to already known species. For instance, new diploid numbers were described for *Ctenomys torquatus* and new fundamental numbers for *Oligoryzomys nigripes* (described as *Oryzomys nigripes* – see references in Table 1). B chromosomes were described for *Sooretamys angouya* and also for four species of *Dasyprocta*. Undescribed rearrangements, including multiple sex chromosome system, were also detected (see Table 1). Moreover, new karyotypes that could not be correlated to any name were published, evidencing the possibility that an undescribed species may exist (*e.g.*: *Akodon* sp. n., *Deltamys* sp., *Thaptomys* sp., *Euryoryzomys* sp., *Neacomys* sp., *Oecomys* sp. 1 – 4, *Oligoryzomys* sp., *Juliomys* sp., *Dasyprocta* sp. *Proechimys* sp. – see Table 1). Additionally (as we will mention below) there are many species with a different diploid number associated that do not represent polymorphisms, which need to be revised (e.g. *Euryoryzomys lamia*, *Euryoryzomys macconnelli*, *Hylaeamys yunganus*, *Oecomys auyantepui*, *Oecomys cleberi*, *Oecomys paricola*, *Oecomys roberti*, *Zygodontomys brevicauda*, *Rhipidomys nitela*, *Phyllomys pattoni*, *Proechimys guyannensis*, etc.).

Since 1984, many species' names have been redescribed or validated (e.g. *Zygodontomys lasiurus* was named as *Bolomys lasiurus* for a long time, and nowadays is

Necromys lasiurus – see synonyms of Table 1). Also, due to the progress of molecular biology during the 1990, associated to morphological information, the number of species described has increased exponentially. It is important to emphasize that molecular phylogeny hitherto has contributed to better understand the cryptic diversity of Brazilian rodents, recognizing monophyletic clades. For instance, new candidate species of *Akodon* (Silva and Yonenaga-Yassuda 1998a, Silva et al. 2006), *Oecomys* (Suárez-Villota et al. under revision), *Oligoryzomys* (Andrades-Miranda et al. 2001a, Miranda et al. 2008), *Neacomys* (Silva et al. 2015, present paper), *Thaptomys* (Ventura et al. 2004, 2010), etc. were recognized based on new karyotypes associated to the monophyly of the samples. Even new genera were described based on multidisciplinary approaches: *Drymoreomys* (Percequillo et al. 2011) and *Calassomys* (Pardiñas et al. 2014).

Technological advances with fluorescent *in situ* hybridization (developed at the end of 1980's but more used during 2000's to date), made it possible to characterize chromosome rearrangements more precisely.

In this paper, we provide a new fundamental number for an undescribed species of *Neacomys*. The karyotype presented here (FN = 66) is similar to the one described by Silva et al. (2015) with FN = 64, except that we found five biarmed pairs and the distribution of constitutive heterochromatin in autosomes was restricted to pericentric regions. We suggest that differences in fundamental numbers are due to pericentric inversions in a small pair, since C-banding evidenced constitutive heterochromatin at the pericentromeric regions, and the morphology of chromosomes was accurately defined. Sex chromosomes presented the same morphology, although the Y was heterochromatic in the short arm (present paper), while it was entirely heterochromatic in the samples described by Silva et al. (2015).

Karyotype information was the first to point out that this specimen may represent a new species, since 2n = 58, FN = 66, has never been described for any *Neacomys* species. Although we used only one molecular marker (incomplete cyt-*b*), which was the same used by Silva et al. (2015), the phylogeny corroborates this information, since all samples with 2n = 58 clustered in a monophyletic high supported the clade. This included two well-supported structured clades, one with samples with FN = 70 (Chaves, Marajó Island) and the other with samples with FN = 64 and 66 (Marabá, Pará State and Vila Rica, Mato Grosso State, respectively), both sister clade to the sample from Igarapé-Açu, Amazonas State. Whether these samples belong to the same undescribed entity with strong population structure or whether they represent at least three different species must be clarified with further phylogeographic and morphological studies, including samples from other localities. This shows the importance of integrative approaches.

In fact, *Neacomys* have a greater diversity than previously known. Recently, based on morphology and molecular phylogeny, Hurtado and Pacheco (2017) demonstrated that *Neacomys spinosus* is a species complex and considered the subespecies *Neacomys spinosus amoenus* a valid species. After this revision, *Neacomys spinosus* is restricted to populations from Peruvian Amazon, and *Neacomys amoenus* encompasses two subspecies: *Neacomys a. amoenus* (from Brazilian Cerrado and Bolivia) and *Neacomys a. carceleni* (from Amazon basin of Ecuador, Brazil and Peru). Thus, sequences related to

N. spinosus from central Brazil, and transition areas of Cerrado and Amazonia correspond to *N. amoenus*. Also, a new species, *N. vargasllosai*, from southern Peru and Bolivia was described. In this same revision, authors recovered three new species pending formal description (the first from Pará, Brazil, the second from Amazonas, Brazil, and the third from Peru and Ecuador). The one from Pará corresponds to the clade composed of samples with 2n = 58 (Fig. 2), reiteraiting the lack of knowledge in this genus.

The description of the karyotype of *Carterodon sulcidens* (a rare species) also corroborates the lack of knowledge for some species, and the importance of fieldwork in discovering new data.

We also show the picture of the karyotype of the exotic species *Mus musculus* for the first time. Despite the noteworthy variation in diploid numbers in Western Europe and Mediterranean populations because of Robertsonian rearrangements (Nachman et al. 1994), in Brazil, the only diploid number described was the standard one (2n = 40).

Progress in cytogenetics: the molecular era

During the beginning of the 1970s (although banding techniques had already been described), karyotypes of Brazilian rodents were studied mainly through conventional staining and the description was limited to diploid and fundamental numbers. Even so, the idea of a wide chromosomal variability already existed. From the 1980s until now, comparative cytogenetics with chromosome banding persists and contributed for elucidating these variations, being that G and C-banding and Ag-NORs are the commonest and cheapest banding techniques.

In fact, the distribution of constitutive heterochromatin and Ag-NORs can be markers in some species. For example, large blocks of constitutive heterochromatin were detected in *Clyomys laticeps* (family Echimyidae) (Souza and Yonenaga-Yassuda 1984, Bezerra et al. 2012) and a huge heterochromatic arm in *Pseudoryzomys simplex* (family Cricetidae, subfamily Sigmodontinae, tribe Oryzomyini) (Moreira et al. 2013). C-band pattern is also an important technique for recognizing sex chromosomes, especially within the subfamily Sigmodontinae (Silva 1994, Di-Nizo 2013). Regarding the nucleolus organizer region, it seems that secondary constriction is a characteristic feature of the family Echimyidae and, as with other vertebrates, may be an important marker. However, chromosomal comparison is now passing from banding patterns to the use of higher resolution innovation of molecular cytogenetics using FISH.

FISH using chromosome painting allows a comparison in a wide genomic scale, revealing a greater number of chromosome changes, unrevealed by the commonest banding techniques, especially in the tribes Akodontini and Oryzomyini of the Subfamily Sigmodontinae. For instance, G-banding pattern showed several rearrangements between *Akodon* species (Tribe Akodontini) (Geise et al. 1998, Silva et al. 2006), but much more complex rearrangements within this genus were observed after cross-species chromosome painting (Ventura et al. 2009).

Extensive chromosomal rearrangements such as Robertsonian, *in tandem* fusion/fission and pericentric inversion, were also observed within the genus *Oligoryzomys*

(Tribe Oryzomyini), after chromosome painting. Using a molecular phylogeny as a reference, it was also possible to detect the direction of the rearrangements and to infer that fission events were as common as fusion events (Di-Nizo et al. 2015). Moreover, Robertsonian rearrangement between *O. rupestris* Weksler & Bonvicino, 2005 (referred as *Oligoryzomys* sp. 1), 2n = 46, FN = 52, and *Oligoryzomys* sp. 2, 2n = 46, FN = 52 was firstly detected by using classic cytogenetic and FISH with telomeric probes (Silva and Yonenaga-Yassuda 1997) and later corroborated by chromosome painting (Di-Nizo et al. 2015). However further studies with molecular phylogeny and morphology are necessary to clarify if both entities represent a single species (with a polymorphism spread in the population) or two different species (in the case of this rearrangement resulted in reproductive incompatibilities leading to the speciation of ancestral population).

The advent of chromosome painting made it possible to compare not only related species but also distant ones, something which is difficult to achieve with banding patterns. Hass et al. (2008) compared *Mus musculus* (family Muridae) to *Akodon* species (family Cricetidae); Nagamachi et al. (2013) compared two different, unrelated genera of the tribe Oryzomiyni (*Cerradomys* and *Hylaeamys*) and Suárez et al. (2015) and Pereira et al. (2016) compared homologies between the tribes Akodontini and Oryzomyini.

Despite the 'modern cytogenetics era', chromosome banding is still an important tool for animal cytogenetic studies, not only because FISH cannot reveal chromosome inversions, but also because it is still a difficult and expensive technique to use.

Chromosome rearrangements and speciation

Rodents proved to be a good model for chromosome evolution studies. Cytogenetics associated with molecular or morphological phylogenetic reconstruction broke cytogeneticist paradigms that fusion rearrangement is more common than fission, and that the reduction in 2n is the expected pattern (e.g. Di-Nizo et al. 2015).

Chromosomal rearrangement could possibly be the cause of reproductive isolation in many Brazilian rodents' species, leading to speciation. The main rearrangements that lead to species formation are Robertsonian, *in tandem* fusion/fission and pericentric inversion, while the variability in constitutive heterochromatin does not seem to create a reproductive barrier and consequent speciation (King 1993, Romanenko and Voloboeuv 2012).

For a long time, it was thought that chromosomal structural rearrangements promoted speciation by generating gametes with duplications and deficiencies, therefore, causing less adaptability of the heterozygotes, but this model was rejected because it lacked theoretical support (Rieseberg 2001, Patton 2004, Jackson 2011). Recently, a different model of chromosome speciation was proposed in which the gene flow is reduced because of recombination-suppression in rearranged regions (Noor et al. 2001, Rieseberg 2001).

In fact, normal meiotic behavior with suppression of crossing over in inverted segments of heteromorphic chromosomes caused by pericentric inversions of *Akodon cursor* and *Oligoryzomys nigripes* was observed, with non-selective disadvantages in het-

erozygous carries (Fagundes et al. 1998, Bonvicino et al. 2001a). Some genetic mechanisms seem to be responsible for overcoming meiotic errors in heterozygous individuals, such as the occurrence of heterosynapsis and the low frequency of chiasm between the inverted segments.

A remarkable chromosome variation can be found in the semi- and fossorial Brazilian rodents *Blarinomys breviceps* (in which molecular phylogeny demonstrated two structured clades – see Ventura et al. 2012), *Clyomys laticeps* and *Ctenomys minutus*. Their species status, and whether their chromosome variation is adaptative and correlated with ecological patterns should be evaluated.

For example, a very well-known case of chromosome speciation due to population adaptation to climatic stress and ecological unpredictability was described in the subterranean rodent *Spalax ehrenbergi* (Family Spalacidae) found in Israel, in which diploid numbers increase coincidently with geographic regions of high aridity (Wahrman et al. 1969). The weak dispersion pattern of this fossorial rodent may have contributed to the fixation of adaptative chromosome change (Árnason 1972).

Cytotaxonomy

Cytotaxonomy is the use of chromosome data as the first clue in the identification of species. Since many Brazilian rodent species present species-specific karyotype and show morphological similarities, chromosome information showed to be useful in the diagnosis of species.

The present revision showed that the delimitation of species based on chromosome data (cytotaxonomy) is essential for recognizing some species of the genera *Akodon*, *Calomys*, *Cerradomys*, *Euryoryzomys*, *Delomys*, *Hylaeamys*, *Juliomys*, *Neacomys*, *Oecomys*, *Oligoryzomys* (family Cricetidae, subfamily Sigmodontinae), *Ctenomys* (family Ctenomyidae), and *Thrichomys* and *Trinomys* (family Echimyidae).

On the other hand, since rates of karyotype evolution differ in distinct branches of the rodents' phylogeny, some species present identical diploid and fundamental numbers, and they cannot be identified solely through chromosome data. This is the case of the following species: (i) *Cavia aperea*, *Cavia fulgida* and *Cavia magna*; (ii) *Kerodon acrobata* and *Kerodon rupestris* (Family Caviidae); (iii) *Akodon lindberghi* and *A. mystax*; (iv) *Akodon paranaensis* and *A. reigi*; (v) *Brucepattersonius griserufescens*, *B. iheringi*, *B. soricinus* and *Thaptomys nigrita*; (vi) *Oxymycterus caparoae*, *Oxymycterus dasytrichus*, *Oxymycterus nasutus* and *Oxymycterus roberti* (the other four species of *Oxymycterus* also have the same diploid number but lacks information on FN) (Family Cricetidae, Subfamily Sigmodontinae, Tribe Akodontini); (vii) *Cerradomys marinhus* and *Pseudoryzomys simplex*; (viii) *Drymoreomys albimaculatus* and *Oecomys* sp. 4; (vix) *Euryoryzomys emmonsae*, *E. nitidus* and *E. russatus* (despite *E. nitidus* and *E. russatus* have disjunction distribution); (x) *Holochilus brasiliensis* and *Nectomys squamipes*; (xi) *Hylaeamys laticeps* and *Hylaeamys seuanezi*; (xii) *Hylaeamys oniscus* and *H. perenensis*; (xiii) *Oecomys bahiensis*, *Oecomys concolor*, *Oecomys* sp. 2 and sp. 3; (xiv) *Neacomys dubosti* and *N. amoenus* (family Cricetidae, Subfamily Sigmodontinae, tribe Oryzomyini); (xv)

Rhipidomys cariri, *R. gardneri*, *R. tribei*, *R. itoan* and *R. macconnelli* (family Cricetidae, Subfamily Sigmodontinae, Tribe Thomasomyini); (xvi) *Dasyprocta azarae*, *D. iacki*, *D. fuliginosa*, *D. leporina*, *D. prymnolopha*, *D. variegata* and *Dasyprocta* sp. (family Dasyproctidae); (xvii) *Isothrix bistriata*, *Mesomys hispidus*, *M. stimulax*, *Trinomys albispinus* and *T. dimidiatus*; (xviii) *Proechimys brevicauda* and *Proechimys cuvieri*; (xix) *Proechimys gardneri* and *Proechimys pattoni* (family Echimyidae) and (xx) *Guerlinguetus brasiliensis* and *Hadrosciurus spadiceus* (family Sciuridae) (Table 1).

Furthermore, some unrelated species, that belong to different tribes, or even families, present the same diploid and fundamental number, suggesting a homoplastic character: (i) *Hylaeamys megacephalus* and *Oxymycterus delator*; (ii) *Juliomys pictipes* and *Thalpomys cerradensis*; (iii) *Calomys laucha* and *Neacomys amoenus* (although there are differences in the size of the biarmed chromosomes); (iv) *Oecomys franciscorum* and *Delomys sublineatus* (despite the first acrocentric pair in *D. sublineatus* is bigger than in *O. franciscorum* as well as the biarmed pair in the last species); (v) *Coendou melanurus* and *Oligoryzomys utiaritensis*; (vi) *Ctenomys ibicuiensis* and *Scolomys ucayalensis* and (vii) *Callistomys pictus*, *Coendou spinosus* and *Myocastor coypus*.

Interdisciplinarity

Since the beginning of the cytogenetic studies in Brazilian rodents, there have been cases in which different karyotypes were assigned to one species or the same karyotype was referred to in different species. In fact, many of these cases were solved after the integration of different disciplines. For instance, for many years cytogenetic information indicated that the previous "*Oryzomys subflavus*" could, in fact, be more than one species, since nine different karyotypes were attributed to a single taxonomic entity (Maia and Hulak 1981, Almeida and Yonenaga-Yassuda 1985, Svartman and Almeida 1992, Silva 1994). Nowadays, after interdisciplinary studies with morphology and molecular phylogeny, it is possible to recognize eight species (Weksler et al. 2006, Percequillo et al. 2008, Tavares et al. 2011, Bonvicino et al. 2014). Moreover, for a long time *Nectomys* was represented by only one species in Brazil, with two diploid numbers (2n = 52 + 1 to 3 Bs and 2n = 56 + 1 to 3 Bs). Nevertheless analyses of the spermatogenesis in hybrids and the sterility of crosses between both cytotypes indicated that *Nectomys* should be considered two distinct species: *Nectomys rattus* (2n = 52) and *Nectomys squamipes* (2n = 56) (Bonvicino et al. 1996).

The opposite occurred in the genus *Oligoryzomys* since the same karyotype (2n = 62, FN = 80-82) was attributed to different names (*O. nigripes*, *O. delticola*, and *O. eliurus*). After molecular and morphology integration, *O. delticola* and *O. eliurus* were considered as a junior synonym of *O. nigripes* (Bonvicino and Weksler 1998).

Some of these cases persist until today, for instance, more than one karyotype was described for *Euryoryzomys macconnelli* and *E. lamia* (Table 1). Molecular phylogeny and morphology corroborate the species complex status of both entities (Almeida 2014, Percequillo 2015a). Similarly, *Oecomys roberti*, *O. paricola*, and *O. catherinae* are probably species complexes, not only because of their variability in diploid number, but

also because of phylogenetic reconstruction and morphological studies (Suárez-Villota et al. 2017). *Ctenomys minutus*, *C. torquatus*, *Hylaeamys yunganus*, *Rhipidomys nitela*, *Sigmodon alstoni* and *Zygodontomys brevicauda* also deserve taxonomic attention because they may represent cases in which different diploid numbers are attributed to the same names. Similarly, *Blarinomys breviceps* has a variable diploid number and two geographic structured clades were recovered in the molecular phylogeny (Ventura et al. 2012), indicating that a morphological revision is needed.

Remarkably, such examples can also be found in the family Echimyidae. The need to use different approaches for taxonomic revision is clear in order to investigate whether *Phyllomys blainvillii*, *Phyllomys pattoni*, and *Proechimys guyannensis* represent species complexes, given the fact that they have more than one karyotype associated.

Interdisciplinary approaches, including cytogenetic, molecular phylogeny, morphology and geographic distribution are essential for accessing the limits of Brazilian rodents' species. One of the best-known examples was the old genera *Oryzomys*, considered the most complex and composing almost half of the species of the tribe Oryzomyini (Musser and Carleton 1993). The current genera *Melanomys*, *Microryzomys*, *Nesoryzomys*, *Oecomys*, and *Oligoryzomys*, were first considered a subgenus of *Oryzomys* and later elevated to the category of genus after morphology, chromosomal and molecular analyses (Myers et al. 1995, Smith and Patton 1999, Bonvicino and Moreira 2001). Another outstanding example of an integrative approach was the study in which ten new genera were described for species that were previously referred to as *Oryzomys* (Weksler et al. 2006), corroborating the cryptic diversity in Oryzomyini previously indicated by cytogenetic data.

Within the Family Echimyidae, the association of morphology and molecular analysis was essential for elevating *Trinomys* (considered subgenus of *Proechimys*) to the genus category (Lara et al. 1996, Leite and Patton 2002).

Perspectives

Despite the new technological approaches, chromosome characterization with conventional staining and banding pattern is still important, mainly because 38 species lack any karyotype information (Table 1). From this amount, 16 are distributed in the Amazonian biome, evidencing the lack of knowledge for this region. The fieldwork is very important and must be encouraged not only because new species and even genera are constantly being described but also because cytogenetic and distribution information of several species are poorly known.

Concerning the family Echimyidae, it is noteworthy that cytogenetic information is lacking for more than 20% of its species. Eleven out of 17 echimyid genera which occur in Brazil are arboreal (Galewski et al. 2005, Emmons et al. 2015). The issues for sampling small arboreal mammals and the consequent low number of studies with this approach have already been highlighted in the literature (Malcolm 1991, Taylor and Lowman 1996, Graipel et al. 2003). In this sense, it can be inferred that this deficiency in echimyid cytogenetic knowledge may be related to sampling scarcity.

The future of molecular biology is promising, with next-generation sequencing (NGS) technology and mitogenomics hopefully providing more robust phylogenetic studies. This new approach was performed with the Family Echymyidae, revealing new supported nodes and clarifying some aspects of the group's taxonomy (Fabre et al. 2016).

However, it is important to reiterate the heterogeneity of characters since DNA, chromosomes, morphology, and behavior are not evolving at the same rate. This particularity may imply in different taxonomic interpretations, with a population being identified as a unique species by one character and two or more species by another, especially in the cases of recent or ongoing speciation. The consequences can be taxonomic inflation or underestimation of the biodiversity, and that is why interdisciplinary approaches are crucial to better understand the biological diversity of rodents.

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Supplementary material 1

Table S1

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Data type: molecular data

Explanation note: Sequences analysed for phylogenetic reconstruction (Maximum likelihood and Bayesian Inference) of *Neacomys*, with species, GenBank and lab/ field number, diploid and fundamental number (when available), locality and reference.

Abbreviations: N/A means that the information is not available. *Cytogenetic data analysed in this work. In bold, sequences obtained in this work. Coordinates for *Neacomys* specimens studied herein: Amazonas State: Igarapé-Açu (04°20'S, 58°38'W); Mato Grosso State: Aripuanã (10°10'S, 59°27"W); Cláudia (11°30'S, 54°53'W); Vila Rica (09°54'S, 51°12'W). Museum and collector acronyms for specimens studied herein: APC (Ana Paula Carmignotto), CIT (Laboratório de Citogenética de Vertebrados - IBUSP), MTR (Miguel Trefaut Rodrigues), MZUSP (Museu de Zoologia, Universidade de São Paulo, Brazil) and PEU (Pedro Luís Bernardo da Rocha).

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