CompCytogen 6(3): 227–237 (2012)
doi: 10.3897/CompCytogen.v6i3.2432
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

A new karyotype for *Rhipidomys* (Rodentia, Cricetidae) from Southeastern Brazil

Ana Heloisa de Carvalho[1], Maria Olímpia Garcia Lopes[2], Marta Svartman[1]

1 *Universidade Federal de Minas Gerais, Laboratório de Citogenética Evolutiva, Departamento de Biologia Geral, Instituto de Ciências Biológicas, Av. Antônio Carlos, 6627 – Pampulha, 31270-910, Belo Horizonte, Brazil* **2** *Biodiversity Salvation, Rua João Pinheiro, 282, Centro, 37925-000, Piumhi, Minas Gerais, Brazil*

Corresponding author: *Marta Svartman* (svartmanm@icb.ufmg.br)

Academic editor: *Jan Zima* | Received 24 November 2011 | Accepted 18 June 2012 | Published 9 July 2012

Citation: de Carvalho AH, Lopes MOG, Svartman M (2012) A new karyotype for *Rhipidomys* (Rodentia, Cricetidae) from Southeastern Brazil. Comparative Cytogenetics 6(3): 227–237. doi: 10.3897/CompCytogen.v6i3.2432

Abstract

In this work we present a new karyotype for *Rhipidomys* Tschudi, 1845 (Cricetidae, Rodentia) from Brazil. Our chromosome analyses included GTG- and CBG-banding patterns, the localization of the nucleolus organizer regions after silver staining (Ag-NORs) and fluorescence *in situ* hybridization (FISH) with a telomere probe. The new karyotype is composed of 44 chromosomes and has a fundamental number (number of autosomal arms) of 48. Most *Rhipidomys* species already karyotyped presented similar complements with 2n=44, but their fundamental numbers varied from FN=46 to 80, a variation that has been mainly attributed to pericentric inversions. The comparison of this new karyotype to those of other *Rhipidomys* already reported allowed us to conclude that it is a distinctive chromosome complement, which can be of great use as a tool for the very complicated taxonomic identification in this genus.

Keywords

Rhipidomys, chromosome banding, FISH

Introduction

The Neotropical rodent *Rhipidomys* Tschudi, 1845 (family Cricetidae) is an arboreal genus belonging to the largely diverse subfamily Sigmodontinae, whose phylogenetic relationships are difficult to resolve, resulting in taxonomic uncertainties at every level, from species to tribes (Musser and Carleton 2005).

Rhipidomys is widely distributed and has been reported from Panama to southeastern Brazil and northern Argentina. The distribution of many species remains uncertain and there are several reports of undescribed species (Tribe 1996, Musser and Carleton 2005). Besides the eighteen species recognized by Musser and Carleton (2005), three additional species have been identified: *R. ipukensis* Rocha et al., 2011, *R. tribei* Costa et al., 2011 and *R. itoan* Costa et al., 2011, and a further unnamed clade from central and eastern Brazil has been recognized (Costa et al. 2011, Rocha et al. 2011). Twelve of the recognized *Rhipidomys* species have been found in Brazil: *R. macconnelli* De Winton, 1900, *R. leucodactylus* Tschudi, 1845, *R. wetzeli* Gardner, 1989, *R. nitela* Thomas, 1901, *R. macrurus* Gervais, 1855, *R. gardneri* Patton et al., 2000, *R. emiliae* J.A. Allen, 1916, *R. mastacalis* Lund, 1840, *R. cariri* Tribe, 2005, *R. ipukensis*, *R. tribei* and *R. itoan*, and an additional undescribed species has been reported as *Rhipidomys* sp 2 (Tribe 1996, Musser and Carleton 2005, Bonvicino et al. 2008, Costa et al. 2011).

Eleven species of *Rhipidomys* have already been karyotyped and, with the exception of *R. nitela* (2n=48) and *Rhipidomys* prope *nitela* (2n=50), all presented karyotypes with 2n=44 chromosomes. In contrast with the conservation of diploid numbers, the karyotypes of *Rhipidomys* showed fundamental numbers ranging from FN=46 to 80, a variation mainly attributed to pericentric inversions. The available karyotypical data for *Rhipidomys* are summarized in Table 1. Most cytogenetic studies on this genus were performed with conventionally stained karyotypes and in less than half the GTG-CBG- or AgNOR-banding patterns were also included.

Table 1. Summary of the available chromosome data for *Rhipidomys*. [1] As *R. sclateri*, which was later considered a synonym of *R. leucodactylus* (Musser and Carleton 2005). [2] Identified by Tribe (1996), originally reported as *Rhipidomys* sp. [3] As *R. cearanus* (Zanchin et al. 1992), later considered as a synonym of *R. mastacalis* (Musser and Carleton 2005).

Group	Species	2n/FN	Locality	Reference
Rhipidomys leucodactylus	*R. leucodactylus*	44/ 46	Rio Juruá (AM)	Patton et al. 2000
		44/ 48	Rio Jamari (RO), Caldas Novas, Serra da Mesa (GO)	Zanchin et al. 1992, Andrades-Miranda et al. 2002
		44/ 48[1]	Cueva del Agua (Venezuela)	Aguilera et al. 1994
		44/ 52	Serra da Mesa (GO), Caxiuanã (PA)	Andrades-Miranda et al. 2002
	Rhipidomus sp.	44/ 48	Berilo (MG)	This work
	R. latimanus	44/ 48	Peñas Blancas (Colômbia)	Gardner and Patton 1976
	R. macrurus	44/ 48	Águas Emendadas (DF), Chapada Diamantina (BA)	Svartman and Almeida 1993, Pereira and Geise 2007
		44/ 49	Granja do Ipê (DF)	Svartman and Almeida 1993
	R. prope *macrurus*	44/ 49[2]	Casa Grande (SP)	Svartman and Almeida 1993
		44/ 50[2]	Monte Verde (ES)	Zanchin et al. 1992
		44/ 50	Garrafão (RJ)	Tribe 1996
		44/ 51	Mocambinho (MG)	Tribe 1996
	R. gardneri	44/ 50	Rio Juruá (AC)	Patton et al. 2000
	R. macconnelli	44/ 50	La Escalera (Venezuela)	Aguilera et al. 1994

Group	Species	2n/FN	Locality	Reference
	R. cf. *mastacalis*	44/ 52	Vila Rica (MT), Aripuanã (MT)	Silva and Yonenaga-Yassuda 1999
	R. itoan	44/ 48,49,50	SP and RJ	Costa et al. 2011
Rhipidomys mastacalis	*R. mastacalis*	44/ 74	Lagoa Santa (MG), Unacau (BA), Casimiro de Abreu (RJ), Reserva Biológica Duas Bocas (ES)	Zanchin et al. 1992, Paresque et al. 1994, Tribe 1996
		44/ 76	Serra da Mesa (GO)	Andrades-Miranda et al. 2002
		44/ 80	Serra da Mesa (GO)	Andrades-Miranda et al. 2002
		³44/ high	Serra dos Cavalos (PE)	Zanchin et al. 1992
Hybrid	*Rhipidomys* with high FN x *Rhipidomys* with low FN	44/ 61	M. Chapéu (BA)	Silva and Yonenaga-Yassuda 1999
Rhipidomys nitela	*R. nitela*	44/ 71	San Ignacio, (Venezuela)	Tribe 1996
		48/ 67	La Trinité (French Guiana)	Volobouev and Catzeflis 2000
		48/ 68	Surumurú (RR)	Andrades-Miranda et al. 2002
	R. prope *nitela*	50/ 71,72	Manaus (AM)	Silva and Yonenaga-Yassuda 1999

In this work, we present a new karyotype for *Rhipidomys*. Our analyses included GTG- and CBG-banding patterns, the silver staining location of the nucleolus organizer regions (Ag-NORs) and fluorescence *in situ* hybridization (FISH) with a telomere probe.

Material and methods

We analyzed five specimens (two males and three females) of *Rhipidomys* sp. captured in 2004 in a dry land region in the margins of the Jequitinhonha river, in Berilo, state of Minas Gerais, Brazil (16°57'06"S, 42°27'56"W; Fig. 1) under the license 129/04-NUFAS/MG from the Instituto Brasileiro para o Meio Ambiente - IBAMA. The skins and skulls were deposited at the Museu de Ciências Naturais da Pontifícia Universidade Católica de Minas Gerais, in Belo Horizonte, Minas Gerais State, Brazil, under the numbers: MCNM 1643, 1644 (two males) and MCNM 1646, 1647, 1648 (three females).

Chromosome preparations were obtained from bone marrow according to the technique described by Ford and Hamerton (1956). GTG- and CBG-banding patterns were obtained following Seabright (1971) and Sumner (1972), respectively, and silver staining of the nucleolus organizer regions (Ag-NORs) was performed according to Howell and Black (1980). FISH with the $(T_2AG_3)_n$ telomere sequence was performed with the Telomere PNA Kit/FITC (Dako Cytomation) according to the manufacturer's instructions.

The chromosomes were arranged based on the karyotype described for specimens of *Rhipidomys* sp. by Svartman and Almeida (1993), which were later identified as *R. macrurus* (Tribe 1996).

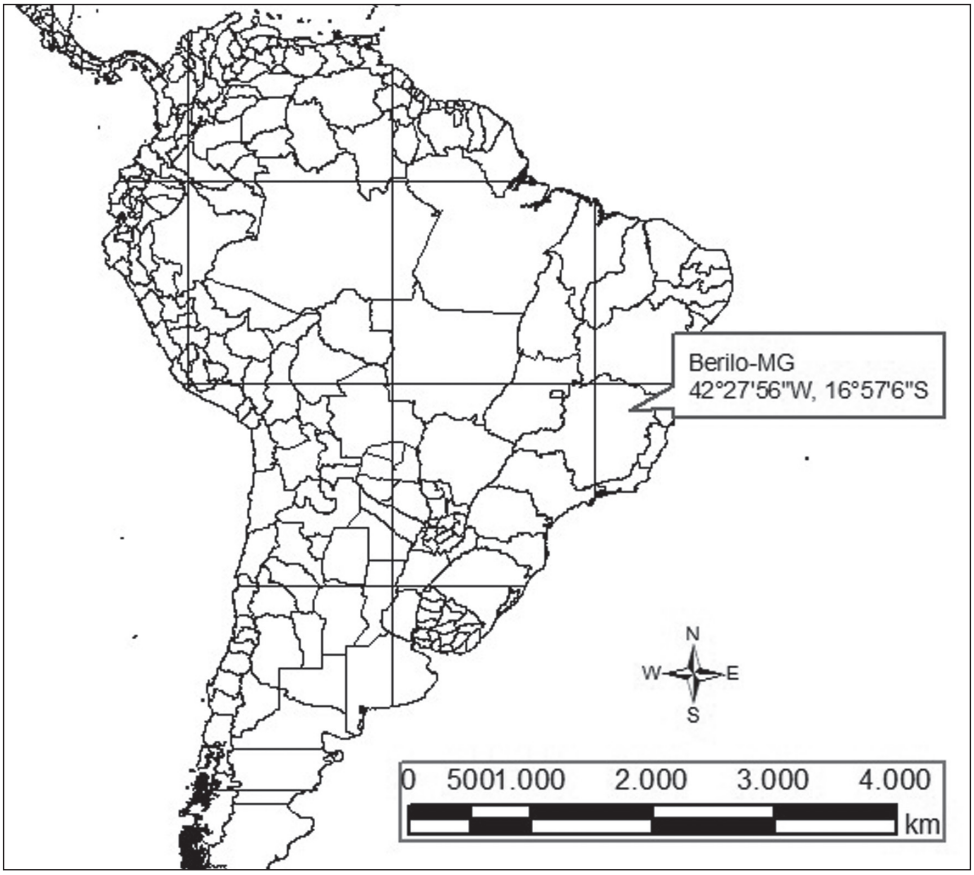


Figure 1. Map showing the collection locality of the *Rhipidomys* sp. analyzed.

Results

The two males and three females of *Rhipidomys* sp. analyzed presented a diploid number of 2n=44 chromosomes and a fundamental number FN=48. This karyotype was composed of 21 pairs of autosomes: 18 pairs of acrocentrics with gradual variation in size from large to small (pairs 1 to 9 and 11 to 19), one pair of medium subtelocentrics (pair 10), one pair of small metacentrics (pair 20) and one pair of small submetacentrics (pair 21). The X chromosome was a large submetacentric with polymorphism in the size of its short arms and the Y chromosome was a very small acrocentric. Autosomal pairs 1, 10, 19, 20 and 21, the X and the Y chromosomes were the only identifiable chromosomes after conventional Giemsa staining (Fig. 2).

After GTG-banding all the autosomes and the sex chromosomes could be identified. The X chromosome presented the two typical mammalian dark GTG-bands in its long arm and no bands were observed on its short arms. The Y chromosome had an indistinct staining (Fig. 3).

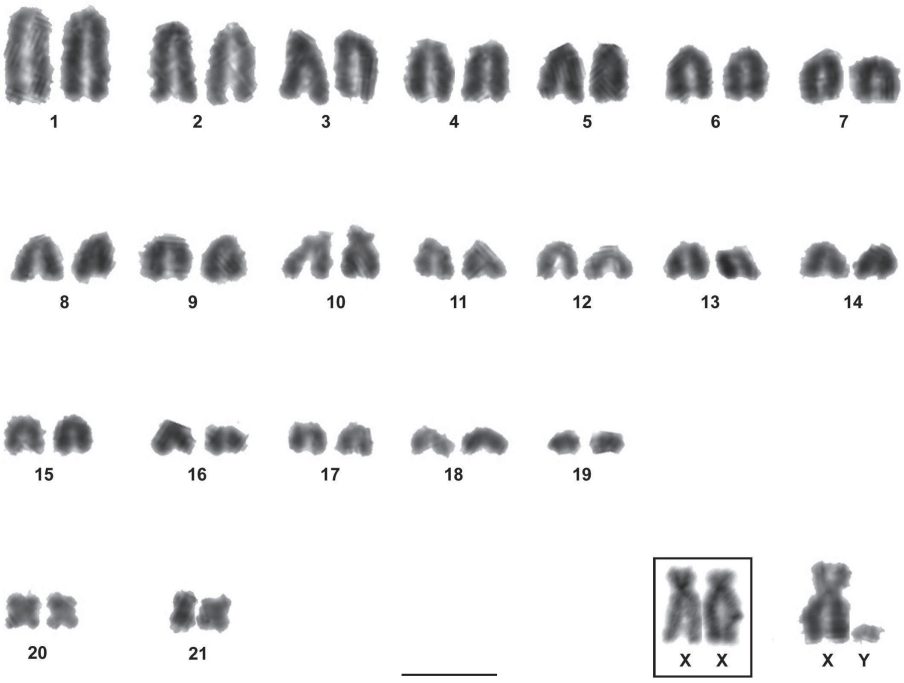


Figure 2. Karyotype of *Rhipidomys* sp. male (2n=44, FN=48) after conventional Giemsa staining. In the inset, the sex chromosomes of a female. Note the variation in the size of the short arms of the X chromosomes. Bar = 10 μm.

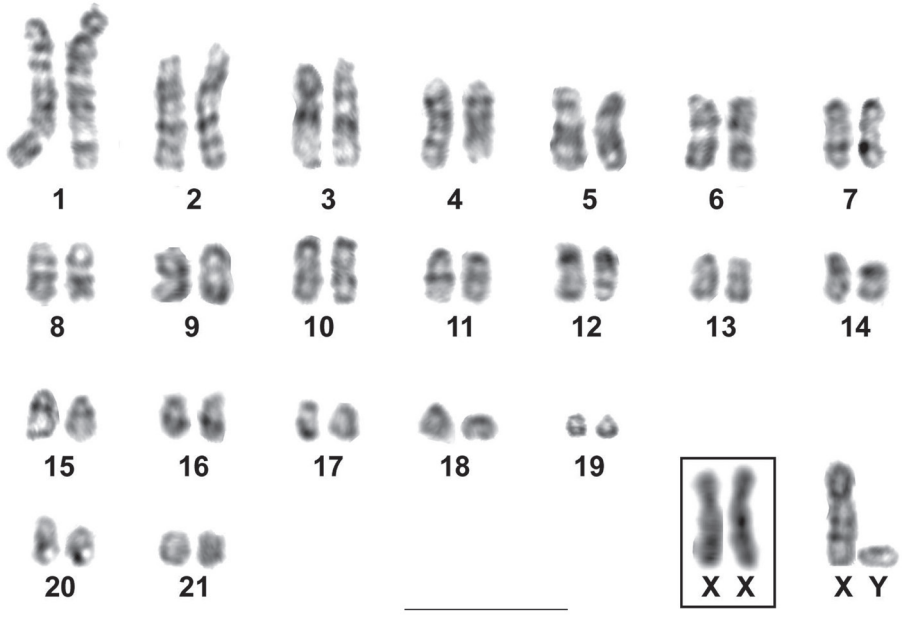


Figure 3. GTG-banded karyotype of *Rhipidomys* sp. male (2n=44, FN=48). In the inset, the sex chromosomes of a female. Bar = 10 μm.

CBG-banding revealed the presence of constitutive heterochromatin in the pericentromeric region of most autosomal pairs. The short arm of the X chromosome was entirely heterochromatic with a stronger stained pericentromeric region and the Y chromosome displayed a small pericentromeric C-band (Fig. 4).

Silver staining revealed one to five nucleolus organizer regions (Ag-NORs) per cell. The Ag-NORs were located on the short arms of medium/small acrocentric autosomes. From the 151 analyzed cells, the majority (57) showed four Ag-NORs. Associations between NORs were frequent (Table 2, Fig. 5). FISH with the telomere sequences revealed signals only at the telomere regions of all chromosomes (Fig. 6).

Figure 4. CBG-banding in a metaphase of *Rhipidomys* sp. female (2n=44, FN=48). Bar = 10 μm.

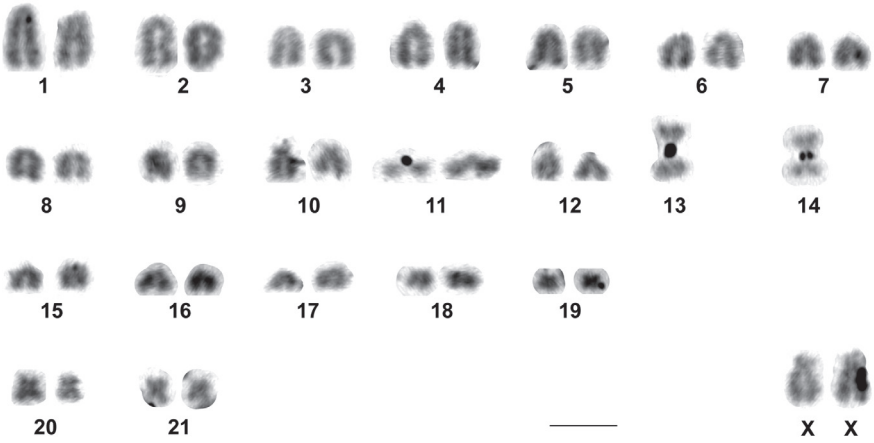


Figure 5. Silver staining of the nucleolus organizer regions (Ag-NORs) in the karyotype of *Rhipidomys* sp. female (2n=44, FN=48). Bar = 10 μm.

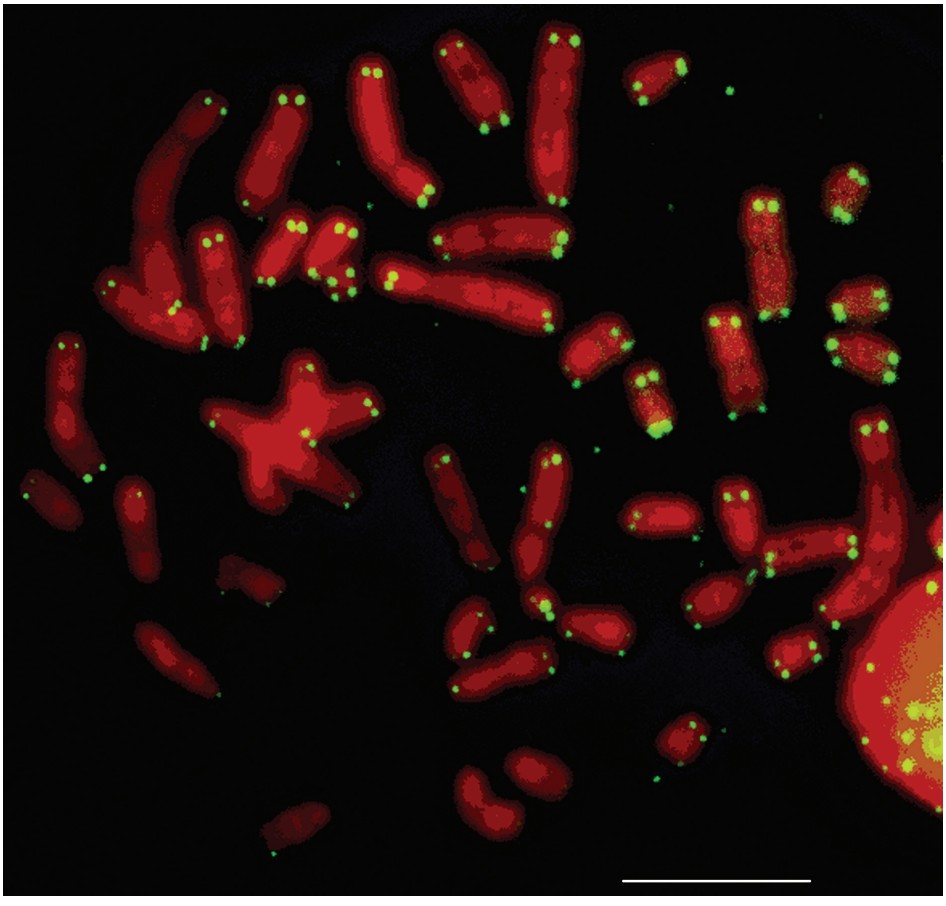


Figure 6. Metaphase of *Rhipidomys* sp. female (2n=44, FN=48) after FISH with a telomere probe. Bar = 10 μm.

Table 2. Number of Ag-NORs per cell in *Rhipidomys* sp. (2n=44, FN=48).

		Number of chromosomes with Ag-NORs					
		1	**2**	**3**	**4**	**5**	**Total**
Number of cells	MCNM 1643 (Male)	4	5	10	10	1	30
	MCNM 1644 (Male)	2	6	6	13	3	30
	MCNM 1646 (Female)	5	4	10	7	4	30
	MCNM 1647 (Female)	1	3	16	11	0	31
	MCNM 1648 (Female)	1	0	12	16	1	29
	Total	13	18	54	57	9	151

Discussion

Besides the karyotype of *Rhipidomys* sp. presented herein, four other species of *Rhipidomys* with the karyotype formula of 2n=44 and FN=48 have been described: *R. latimanus* Tomes, 1860 from Colombia, for which no karyotype picture has been presented (Gardner and Patton 1976); *R. macrurus* from the Brazilian states of Goiás and Bahia (Svartman and Almeida 1993, Pereira and Geise 2007), *R. leucodactylus* collected in the Brazilian states of Rondônia and Goiás (Zanchin et al. 1992, Andrades-Miranda et al. 2002) and in Venezuela (Aguilera et al. 1994), and *R. itoan* from the states of São Paulo and Rio de Janeiro. The animals from Venezuela were originally identified as *R. sclateri* (Aguilera et al. 1994), which was later recognized as a synonym of *R. leucodactylus* (Musser and Carleton 2005).

The karyotype of *Rhipidomys* sp. studied herein differed from that of *R. macrurus* (2n=44, FN=48) from Goiás (Svartman and Almeida 1993) in the morphologies of pair 10 and of the smallest autosome pair. Pair 10 was subtelocentric in *Rhipidomys* sp. and acrocentric in *R. macrurus*, whereas the smallest autosome pair was acrocentric in *Rhipidomys* sp. and metacentric in *R. macrurus*. The X chromosome was submetacentric in *R. cariri* and acrocentric in *R. macrurus*. The CBG-banding patterns also differed between both species, as only a very small amount of constitutive heterochromatin was detected in *R. macrurus* (Svartman and Almeida 1993), compared to the pericentromeric C-bands found in most autosomes of *Rhipidomys* sp. (Fig. 3). Silver staining revealed the presence of 1 to 5 chromosomes with nucleolus organizer regions (Ag-NORs) in *Rhipidomys* sp. All the NOR-bearing chromosomes were medium acrocentrics similar to the three pairs that presented Ag-NORs in *Rhipidomys macrurus* (Svartman and Almeida 1993).

GTG-banding patterns have not been described for *R. leucodactylus*, also with 2n=44 and FN=48. From the three biarmed autosomes found in the karyotype of this species, two are comparable in size to the medium acrocentric pair 15 and the third is the smallest autosome pair (Zanchin et al. 1992, Aguilera et al. 1994, Andrades-Miranda et al. 2002), whereas in *Rhipidomys* sp. the biarmed chromosomes correspond in size to pairs 10, 15 and 16. The X chromosome also differed between both species, being biarmed in *R.* sp. and acrocentric in *R. leucodactylus*. Interestingly, the complement of *R. leucodactylus* seemed identical to that of *R. macrurus* from Goiás (Svartman

and Almeida 1993), but the absence of GTG-banding patterns of *R. leucodactylus* in the literature hindered further comparisons.

In *Rhipidomys itoan* with 2n=44 and FN=48 the smallest autosome pair was a submetacentric (Costa et al. 2011), differing from the acrocentric smallest autosome of *R.* sp. presented herein. Morphological variations were observed in two large pairs of *R. itoan*, that could be acrocentric or biarmed, leading to higher fundamental numbers (FN=49 and 50). No such variation was detected in our specimens.

The absence of banding patterns descriptions limited the comparisons of the complement of *R.* sp. described in this work and those of *R. itoan* and *R. leucodactylus* to conventionally stained chromosomes.

The *Rhipidomys* species already recorded in Minas Gerais were *R. macrurus*, which is probably distributed in the remaining Cerrado fragments of the state, *R. mastacalis*, which was collected in the Atlantic Forest in eastern and southern Minas Gerais, and *R. tribei*, known from only a few sites in the southeastern part of Minas Gerais (Tribe 1996, Bonvicino et al. 2008; Costa et al. 2011).

Rhipidomys mastacalis is characterized by a high fundamental number (FN=74 through 80) (Zanchin et al. 1992, Paresque et al. 1994, Tribe 1996, Andrades-Miranda et al. 2002) which promptly allows to differentiate its karyotype from that of *Rhipidomys* sp. (FN=48). On the other hand, *R. macrurus* (FN=48-50; variation due to polymorphism in the morphology of pair 10) (Svartman and Almeida 1993, Pereira and Geise 2007) presented a complement very similar to that of *Rhipidomys* sp. (FN=48). Nevertheless, as discussed above, the two karyotypes differ in the morphology of the smallest autosome pair (pair 19, acrocentric in *Rhipidomys* sp., and pair 21, metacentric in *R. macrurus*), and in the amount of constitutive heterochromatin, which can thus be used to differentiate both species. No chromosome data are available for *R. tribei*.

FISH with telomere sequences has been previously performed in specimens of *R. nitela*, *R. mastacalis* and *R. leucodactylus* (Andrades-Miranda et al. 2002), *R.* prope *mastacalis* and in animals of two unidentified species (Silva and Yonenaga-Yassuda 1999). As for *Rhipidomys* sp. presented herein, only terminal signals were observed in the cells of all these specimens. Interstitial signals, which could give clues on chromosome rearrangements, have not been observed in *Rhipidomys* as yet.

The identification of *Rhipidomys* specimens from southeastern Brazil at the species-level has proven to be specially challenging, with *R. macrurus* and *R. mastacalis* being among the most taxonomically complicated taxa studied (Musser and Carleton 2005; Tribe 1996, Costa et al. 2011).

Chromosome analyses may be useful for the identification of species, especially in complicated taxonomic groups, as is the case of many rodent taxa. As stressed by Tribe (1996), the use of non-morphological characters, as karyotypes, allozymes and DNA sequences, may help in clarifying the phylogenetic relationships and in the taxonomic identification of *Rhipidomys* species. This prediction has proven right in works like those of Costa et al. (2011) and Rocha et al. (2011), which used molecular data to further the knowledge of the genus, resulting in the description of new species and in the clarification of some phylogenetic relationships. Likewise, karyotypical data, especially

those including banding patterns, are likely to add new information and to help in clarifying the taxonomy and phylogenetics of this intriguing rodent genus.

With the available data, it seems evident that a larger collection effort including a wider geographical range and complemented by cytogenetic and molecular studies will be needed in order to establish the phylogenetic relationships and phylogeography of *Rhipidomy*s in Brazil. Nevertheless, as exemplified in this work, the use of chromosome data has already proven to be a useful tool in resolving taxonomic issues in this genus.

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Fapemig (processes CBB APQ-1495-3.12/07, CRA - APQ-00170-09 and APQ-00336-09) and by a grant of the Pró-Reitoria de Pesquisa, UFMG. AHC was a recipient of a graduate fellowship from CAPES, Brazil. The authors are indebted to the Laboratório de Citogenética, Hospital das Clínicas, UFMG, for the use of their laboratory and to Bárbara MA Costa, for the useful discussion of the manuscript.

References

Aguilera M, Pérez-Zapata A, Martino A, Barros MA, Patton J (1994) Karyosystematics of *Aepeomys* and *Rhipidomys* (Rodentia, Cricetidae). Acta Científica Venezoelana 45: 247–248.

Andrades-Miranda J, Oliveira LFB, Lima-Rosa CAV, Sana DA, Nunes AP, Mattevi MS (2002) Genetic studies in representatives of genus *Rhipidomys* (Rodentia, Sigmodontinae) from Brazil. Acta Theriologica 47: 125–135. doi: 10.1007/BF03192453

Costa BMA, Geise L, Pereira LG, Costa LP (2011) Phylogeography of *Rhipidomys* (Rodentia, Cricetidae: Sigmodontinae) and description of two new species from southeastern Brazil. Journal of Mammalogy 92: 945–962. doi: 10.1644/10-MAMM-A-249.1

Bonvicino CR, Oliveira JA, D'Andrea OS (2008) Guia dos Roedores do Brasil, com chaves para gêneros baseadas em caracteres externos. Centro Pan-Americano de Febre Aftosa - OPAS/OMS, Rio de Janeiro, 122 pp.

Ford CE, Hamerton JL (1956) A colchicine hypotonic citrate squash sequence for mammalian chromosome. Stain Technology 31: 247–251.

Gardner AL, Patton JL (1976) Karyotypic variation in Oryzomyini rodents (Cricetinae) with comments on chromosomal evolution in the Neotropical cricetinae complex. Occasional Papers of the Museum of Zoology LA State University 9: 1–48.

Howell WM, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36: 1014–1015. doi: 10.1007/BF01953855

Musser GG, Carleton MD (2005) Family Muridae. In: Wilson DE, Reeder DM (Eds) Mammal Species of the World. A Taxonomic and Geographic Reference. Baltimore, 3rd edition, 1189–1531.

Paresque R, Souza WP, Mendes S, Fagundes V (2004) Composição cariotípica da fauna de roedores e marsupiais de duas áreas de Mata Atlântica do Espírito Santo, Brasil. Boletim do Museu de Biologia Mello Leitão 17: 5–33.

Patton JL, da Silva MNF, Malcolm RJ (2000) Mammals of Rio Juruá, Western Amazonian Brazil: Evolutionary and Ecological Diversification. Bulletin American Museum Natural History 244: 1–306. doi: 10.1206/0003-0090(2000)244<0001:MOTRJA>2.0.CO;2

Pereira LG, Geise L (2007) Karyotype composition of some rodents and marsupials from Chapada Diamantina (Bahia, Brasil). Brazilian Journal of Biology 67: 509–518. doi: 10.1590/S1519-69842007000300016

Rocha RG, Ferreira E, Costa BMA, Martins ICM, Leite YR, Costa LP, Fonseca C (2011) Small mammals of the mid-Araguaia River in Central Brazil, with the description of a new species of climbing rat. Zootaxa 2789: 1–34.

Seabright M (1971) A rapid technique for human chromosomes. Lancet 2: 971–972. doi: 10.1016/S0140-6736(71)90287-X

Silva MJJ, Yonenaga-Yassuda Y (1999) Autosomal and sex chromosomal polymorphisms with multiple rearrangements and a new karyotype in the genus *Rhipidomys* (Sigmodontinae, Rodentia). Hereditas 131: 211–220. doi: 10.1111/j.1601-5223.1999.00211.x

Smith M, Patton JL (1999) Phylogenetics relationships and the radiation of Sigmodontinae rodents in South America: Evidence from cytochrome b. Journal of Mammalian Evolution 6: 89–128. doi: 10.1023/A:1020668004578

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75: 305–306. doi: 10.1016/0014-4827(72)90558-7

Svartman M, Almeida EJC (1993) Pericentric inversion and X chromosome polymorphism in *Rhipidomys* sp. (Cricetidae, Rodentia) from Brazil. Caryologia 46: 219–225.

Tribe CJ (1996) The Neotropical rodent genus *Rhipidomys* (Cricetidae, Sigmodontinae) – a taxonomic revision. PhD Dissertation, University College, London, 316 pp.

Volobouev VT, Catzeflis FM (2000) Chromosome banding analysis (G-, R- and C-bands) of *Rhipidomys nitela* and a review of the cytogenetics of *Rhipidomys* (Rodentia, Sigmodontinae). Mammalia 64: 353–360. doi: 10.1515/mamm.2000.64.3.353

Zanchin NIT, Langguth A, Mattevi MS (1992) Karyotypes of Brazilian species of *Rhipidomys* (Rodentia, Cricetidae). Journal of Mammalogy 73: 120–122. doi: 10.2307/1381872

CompCytogen 6(3): 239–247 (2012)
doi: 10.3897/CompCytogen.v6i3.3261
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Sex chromosome differentiation in *Humulus japonicus* Siebold & Zuccarini, 1846 (Cannabaceae) revealed by fluorescence *in situ* hybridization of subtelomeric repeat

Oleg S. Alexandrov[1], Mikhail G. Divashuk[1], Nikolay A. Yakovin[1], Gennady I. Karlov[1]

1 *Centre for Molecular Biotechnology, Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Moscow 127550, Timiryazevskaya Street, 49, Russia*

Corresponding author: *Gennady Karlov* (karlovg@gmail.com)

Academic editor: *Lorenzo Peruzzi* | Received 20 April 2012 | Accepted 27 June 2012 | Published 10 July 2012

Citation: Alexandrov OS, Divashuk MG, Yakovin NA, Karlov GI (2012) Sex chromosome differentiation in *Humulus japonicus* Siebold & Zuccarini, 1846 (Cannabaceae) revealed by fluorescence *in situ* hybridization of subtelomeric repeat. Comparative Cytogenetics 6(3): 239–247. doi: 10.3897/CompCytogen.v6i3.3261

Abstract

Humulus japonicus Siebold & Zucc (Japanese hop) is a dioecious species of the family Cannabaceae. The chromosome number is 2n = 16 = 14 + XX for females and 2n = 17 = 14 + XY1Y2 for male. To date, no fluorescence *in situ* hybridization (FISH) markers have been established for the identification of *H. japonicus* sex chromosomes. In this paper, we report a method for the mitotic and meiotic sex chromosome differentiation in *H. japonicus* by FISH for HJSR, a high copy subtelomeric repeat. The signal is present in the subtelomeric region of one arm of the X chromosome. We demonstrate that males have two Y chromosomes that differ in FISH signal with the HJSR probe. Indeed, the HJSR probe hybridizes to a subtelomeric region on both arms of chromosome Y1 but not of chromosome Y2. The orientation and position of pseudoautosomal regions (PAR1 and PAR2) were also determined.

Keywords

Humulus japonicus, sex chromosomes, sex determination in plants, subtelomeric repeat, fluorescence *in situ* hybridization

Introduction

Humulus japonicus Siebold & Zuccarini, 1846 (Japanese hop) is a dioecious, climbing and annual species of the family Cannabaceae. The chromosome number is 2n = 16 = 14 + XX for females and 2n = 17 = 14 + XY1Y2 for males. The sex of *H. japonicus* is

determined by the ratio of X chromosomes and autosomes sets (A); a X:A ratio of 1.0 results in a female and a ratio of 0.5 results in a male (the Y chromosomes are dispensable) (Bridges 1921, Parker and Clark 1991, Shephard et al. 2000, Ming et al. 2007, Ming et al. 2011). The Y chromosomes in *H. japonicus* are markedly heterochromatic (Shephard et al. 2000). The multiple sex chromosome system (XX/XY1Y2) is similar to that of *Rumex acetosa* Linnaeus 1753, a model species in studies of sex determination and sex chromosome organisation in plants (Ruiz Rejón et al. 1994).

The closest relative of *H. japonicus* is the common hop *Humulus lupulus* Linnaeus, 1753. *H. lupulus* has the same sex determination system as in Japanese hop (X/A) but it differs in chromosome number (2n = 20 in both female and male plants) and in sex chromosome systems (XX/XY) (Winge 1929, Ono 1955, Shephard et al. 2000). Molecular phylogenetic analyses of cpDNA and nuclear rDNA coding regions in *H. lupulus* and *H. japonicus* have revealed the high similarity of these two species. The time of divergence between these two species was estimated to be 6.38 million years ago (Murakami et al. 2006).

Due to the great economic importance of *H. lupulus*, molecular methods to assess genetic variability and genome organisation have been developed for this species. To understand sex chromosome evolution and organisation in plants, sex-linked genetic and cytogenetic markers are required. Male-specific DNA markers have been identified in *H. lupulus* (Polley et al. 1997, Seefelder et al. 2000, Danilova and Karlov 2006, Jakse et al. 2008) and recently in *H. japonicus* (Alexandrov et al. 2011).

Cytogenetic markers of *H. lupulus* sex chromosomes were established by application of C-banding/DAPI (Karlov et al. 2003) and FISH with a subtelomeric repeat (HSR1) as a probe (Divashuk et al. 2011). Cytogenetic analysis of *H. japonicus* has been limited, and little is known about the molecular cytogenetic organisation of the *H. japonicus* sex chromosomes. After conventional staining, autosomes and sex chromosomes cannot be morphologically distinguished from each other. Recently, molecular characterisation of the *H. japonicus* karyotype was completed by Kim et al. (2008) and Grabowska-Joachimiak et al. (2011). Using telomere repeats, 5S and 45S rDNA probes and C-banding/DAPI staining, a fluorescent karyotype was constructed. The latter study demonstrated that sex chromosomes of *H. japonicus* display unique DAPI banding patterns. The X chromosome possesses only one brightly stained AT-rich terminal segment, while Y1 has 2 such segments, and Y2 have no DAPI positive signal. This distribution of signal and the large size of the sex chromosomes allowed the authors to distinguish them from the autosomes and each other (Grabowska-Joachimiak et al. 2011).

A trivalent formation comprising Y1-X-Y2 associated with terminal chiasmata has been observed during meiosis in *H. japonicus* (Shephard et al. 2000, Kim et al. 2008). However, these observations were made without the benefit of cytogenetic markers for sex chromosomes, and nothing is known about their orientation in the trivalent formation.

To date, no FISH markers have been established for the identification of *H. japonicus* sex chromosomes. In this paper, we report a method for sex chromosome differentiation in *H. japonicus* by FISH with the subtelomeric repeat HJSR on mitotic and meiotic chromosomes.

Material and methods

Male and female plants of *Humulus japonicus* raised from seeds of cv. Samuray ("Gavrish seeds", Moscow, Russia) and seed lot № 4 ("Flos", Moscow, Russia) were used in this study.

Total genomic DNA was isolated from young leaf material using the CTAB method (Rogers and Bendich 1985). To isolate the *H. japonicus* subtelomeric repeat HJSR, the DNA was digested by various restriction enzymes (*Alu*I, *Dra*I, *Eco*RI, *Hin*6I, *Hin*cII, *Hind*III, *Kpn*I, *Not*I, *Pst*I, *Xmi*I, *Bc*I, *Hae*III, *Vha*464I, *Bam*HI, *Nco*I, *Taq*I). The bands obtained after gel electrophoresis were carefully cut out from the gel, and the DNA was eluted with the QIAquick Gel Extraction Kit. The cloning of the eluted DNA was performed with the pUC 19 vector. Nucleotide sequences were determined using an ABI 3130 XL (Applied Biosystems) after sequencing reactions with a Big Dye Terminator v 1.1. Cycle Sequencing Kit (Applied Biosystems). BLAST analysis was performed according to standard procedures.

Mitotic metaphase chromosomes were prepared from fast growing root tip meristems collected from plants. They were pre-treated in 0.01 % α-bromonaphtalene at 4°C for 24 h and fixed in 3:1 (v/v) 96% ethanol: glacial acetic acid at room temperature for 1 h. For preparation of the microscopic slides, the root tips were rinsed in running water for 1 h and in distilled water three times and then were incubated in a 10 mM citrate buffer (pH 4.9) containing 0.4 % cellulase Onozuka R10 (Serva, Germany) and 0.2 % pectolyase Y-23 at 37°C for 3 h. Afterwards, the macerated root tips were spread by dissecting the tissue in 60 % acetic acid and by squashing it under a coverslip.

For meiotic chromosome preparations, the young anthers about 3-5 mm long at metaphase I were collected and fixed directly in acetic-ethanol (1:3) for 1 h, rinsed in water and then incubated for 2–3 hours in pectolytic enzymes containing 0.8 % cellulase Onozuka R10 (Serva, Germany) and 0.4 % pectolyase Y-23 in a 10 mM citrate buffer (pH 4.9). After two washes in distilled water, the anthers were carefully transferred onto grease-free slides, and the pollen mother cells were dissected out of the anther into a 1 μl droplet of water. Then, 5 μl of 60 % acetic acid was added, and the pollen mother cells were left for 2–3 minutes until the cytoplasm became sufficiently clear. The cells were then squashed under a coverslip.

For fluorescence *in situ* hybridization (FISH), the plasmid with the *H. japonicus* HJSR subtelomeric repeat DNA was labelled with dioxigenin-11-dUTP. The 1 μg sample of the purified DNA was labelled by nick translation according to the manufacturer's protocol (Roche Diagnostics Gmbh, Germany). The chromosome and probe denaturation as well as hybridization and posthybridization washes were performed as described by Karlov et al. (2003). The chromosome preparations were counterstained with 5 μg/ml propidium iodide and mounted in Vectashield (Vector Laboratories, UK).

For detection of *Arabidopsis*-type telomere repeat in *H. japonicus* chromosomes sequential FISH was applied. Cover glasses were carefully removed after by washing for 1 h with 0.2 % Tween 20. Probe DNA was dissociated from the chromosomes with 70 % formamide in 2×SSC for 5 min. Slides were the dehydrated for 3 min each of 70,

90 and 100 % (v/v) ethanol, and air-dried. A new hybridization mix was added to the slides. The *Arabidopsis*-type telomere probe used was the deoxyribinucleotide oligomer $(5'\text{-CCCTAAA-}3')_3$ synthesised with a TAMRA label (ZAO "Syntol", Moscow, Russia) at the 5' end. The chromosome preparations were counterstained with DAPI.

The slides were observed under an AxioImager.M1 fluorescent microscope, photographed with a monochrome AxioCam MRm CCD camera, and visualised using Axiovision software (Carl Zeiss). In each experiment, at least 35 chromosome plates were analysed.

Results

The isolated and cloned HJSR *Kpn*I-repeat was sequenced and found to be 380 bp in length (GU831573). No sex specific differences have been found between the sequences of male and female plants. The consensus sequence of 380 bp fragment is 63.4 % AT and does not possess any direct or inverted sequences of significant length. The BLAST analysis did not reveal any significant homology with sequences of other organisms.

The FISH signals observed with the HJSR probe were localised to subtelomeric regions of the chromosomes, and the signals were observed at one or both distal ends of each chromosome in both males and females. However, the signal was completely absent on one pair of autosomes from males and females and additionally on one of the three biggest chromosomes from males (Fig. 1). The FISH signal colocalised with the subtelomeric DAPI positive bands. No signal was detected from the interstitial regions of the chromosomes. The metaphase plates of the male and female plants were compared and revealed that the female metaphase plates carry two X chromosomes with the HJSR repeat signal on one of the arms (Fig. 1a, c). The male metaphase plates appeared to possess chromosome X with one signal, chromosome Y1 with signals on both arms and chromosome Y2 with no signal (Fig. 1d, f). FISH of the mitotic chromosomes of *H. japonicus* with a probe for an *Arabidopsis*-type telomere repeat showed signals on the all chromosome ends of both male and female plants (Fig 1b, e). The locations of the FISH signals from the telomeric probe were more distal from the centromere than those with the HJSR probe. No interstitial *Arabidopsis*-type telomere repeat signals were observed on the chromosomes.

The results of the mitotic metaphase plate analyses are in agreement with the physical mapping of the HJSR to the meiotic chromosomes at diakinesis (Fig. 2). We identified the Y1-X-Y2 trivalent formation (Fig. 2a, b). The different ends of the X chromosome pair with different Y chromosomes. The Y1 chromosome, revealed HJSR FISH signals on both arms, pairs with arm of the X chromosome also carrying HJSR FISH signal. The Y2 chromosome has no HJSR FISH signal and pairs with the X chromosome arm that lacks a signal. This finding allows us to conclude that the pseudoautosomal regions (PAR1 and PAR2) are located at distal parts of both arms of the X chromosome and distally on one arm of each Y chromosome (Fig. 2c).

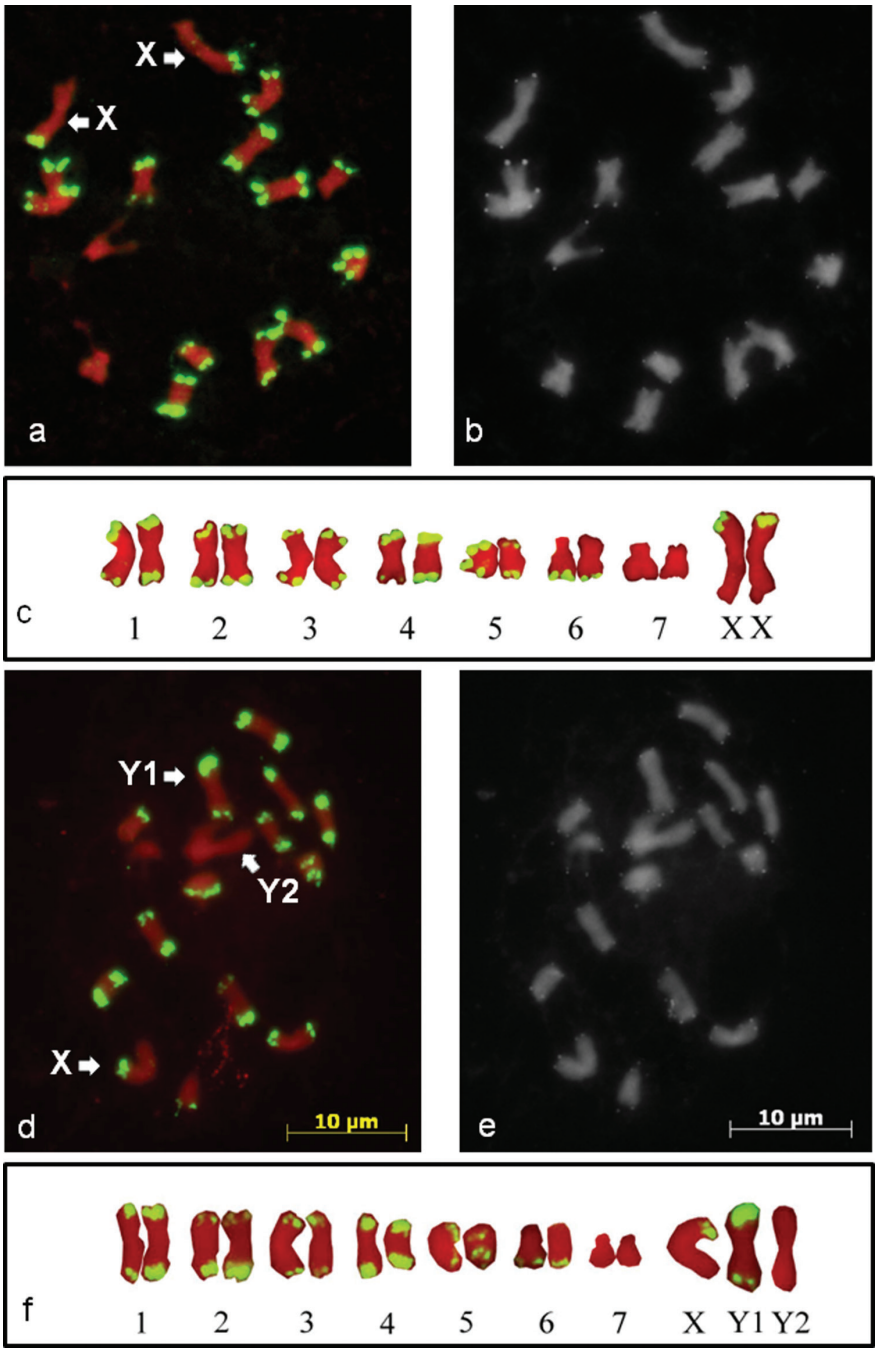


Figure 1. The mitotic chromosomes of *H. japonicus*. The chromosomes are counterstained by propidium iodide (red). The high copy HJSR subtelomeric repeat (green) is mapped to the female **(a)** and male **(d)** mitotic chromosomes of *H. japonicus* by FISH. The X, Y1 and Y2 chromosomes are marked by arrows. Sequential FISH with the *Arabidopsis*-type telomeric repeat on metaphase chromosomes of female (**b**) and male plants **(e).** The katyotypes of female **(c)** and male **(f)** plants.

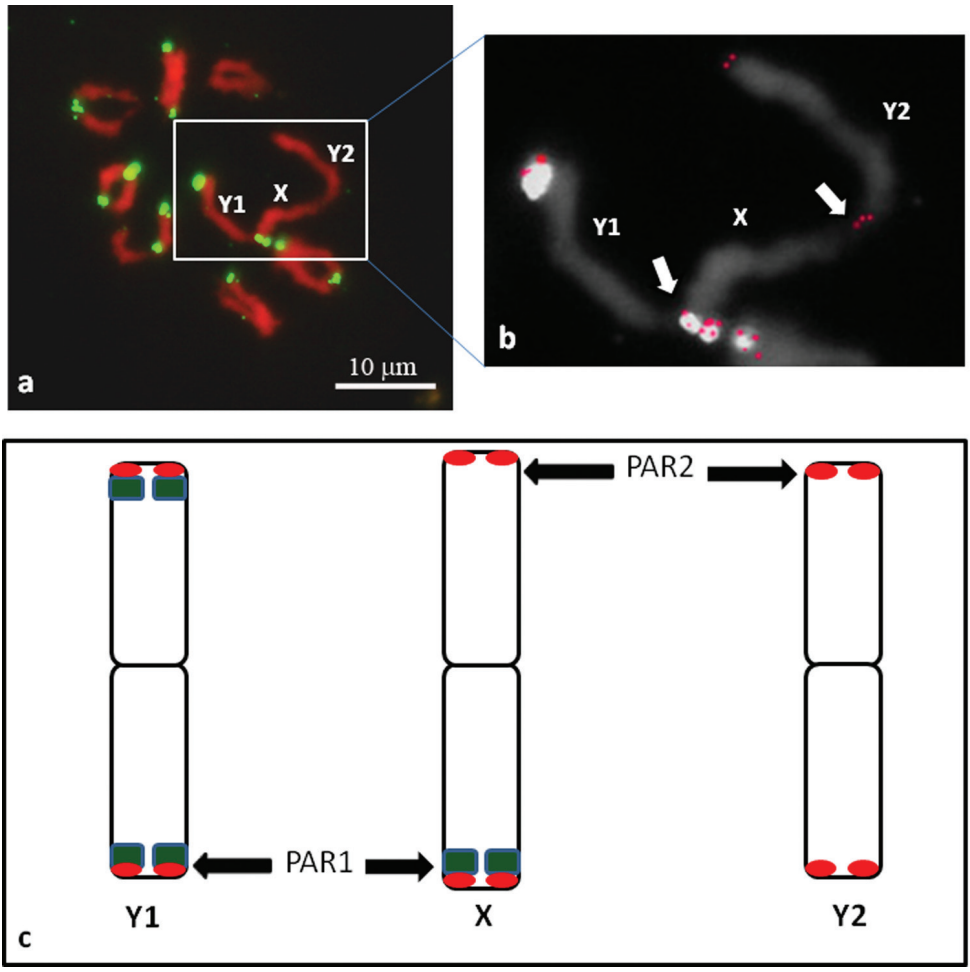


Figure 2. The meiotic chromosomes of *H. japonicus* at diakinesis with FISH signals for the HJSR repeat (green). The trivalent Y1-X-Y2 formation and chiasmata between the sex chromosomes can be clearly observed **(a).** The trivalent Y1-X-Y2 formation from **(a)** with combined signal of *Arabidopsis*-type telomeric repeat after sequential FISH (red) **(b)**. Schematic diagram of the *H. japonicus* X, Y1 and Y2 chromosomes **(c)** with the hybridization of the HJSR probe (green) and the *Arabidopsis*-type telomeric repeat probe (red). The pseudoautosomal regions (PAR1 and PAR2) are indicated by the arrows.

Discussion

Most satellite DNAs are specific at the species or species subgroup levels (Kazama et al. 2003). Their presence and distribution reflect evolutionary events (Kubis et al. 1998, Koo et al. 2010). In this study, we isolated and described for the first time a new satellite DNA subtelomeric repeat, HJSR. This satellite DNA is localised at subtelomeric positions and colocalises with DAPI positive bands, except for the interstitial DAPI positive bands on chromosomes 3 and 7 discovered by Grabowska-Joachimiak et al. (2011). This signal pattern is in agreement with the DAPI staining, which detects AT-

rich regions such as HJSR (63.4 %). The absence of this repeat in interstitial DAPI bands of chromosomes 3 and 7 indicates the presence of another type of AT-rich repeat. Interestingly, there are differences between the chromosomes with subtelomeric HJSR repeats on one or both arms, and the distribution of signal on the sex chromosomes is also different. These observations suggest the occurrence of chromosome reorganisation, implying that duplications or deletions may have occurred. In the common hop (*Humulus lupulus* L.), a *Kpn*I species specific subtelomeric repeat (HSR1) has also been cloned (Divashuk et al. 2011). The chromosome organisation of *H. lupulus* X chromosomes, as assessed by FISH for a subtelomeric repeat, was different from that of *H. japonicus*. Unlike *H. japonicus, H. lupulus* X chromosomes contain an interstitial HSR1 subtelomeric repeat site near the centromere. This difference reveals a karyotype reorganisation and sex chromosome evolution among these two closely related species. This also explains the different number and position of 5S and 45S rDNA loci on the autosomes of these two species (in *H. lupulus* 2 and 1, and in *H. japonicus* 1 and 2 loci, respectively) (Karlov et al. 2003, Kim et al. 2008, Grabowska-Joachimiak et al. 2011). Also, a higher amount of nuclear DNA has been found in *H. lupulus* (2C = 5.6 pg vs. 3.2 pg) (Grabowska-Joachimiak et al. 2006). The lower DNA content in *H. japonicus* may be due to the loss of subtelomeric repeats, as shown in our study for one pair of autosomal chromosomes and chromosome Y2. In contrast, all chromosomes of *H. lupulus* have subtelomeric *Kpn*I-repeats (Divashuk et al. 2011).

The orientation of the pseudoautosomal regions on the X chromosome indicates the important role of subtelomeric repeats in sex chromosome genesis. The nature of the Y chromosomes of *Humulus* is puzzling. The unusual sex chromosome system XX/XY1Y2 in *H. japonicus* points to the role of chromosome translocations in the karyotype evolution of this species. According to Ohno's (1967) hypothesis, multiple sex chromosomes have evolved from the standard XX/XY systems by interchanges between autosomes and sex chromosomes. This has been shown in *Silene diclinis* (Lag.) M. Laínz, 1963 (Howell et al. 2009) and may also have occurred in *H. japonicus*.

Acknowledgements

This work was supported by grant # P1164 NK-602P/7 from the Russian Ministry of Science and Education.

References

Alexandrov OS, Divashuk MG, Karlov GI (2011) Development of sex specific DNA marker for Japanese hop *Humulus japonicus* Siebold & Zucc. Russian Journal of Genetics 47(8): 1016–1020.

Bridges CB (1921) Triploid Intersexes in *Drosophila melanogaster.* Science 54(1394): 252–254. doi: 10.1126/science.54.1394.252

Danilova TV, Karlov GI (2006) Application of inter simple sequence repeat (ISSR) polymorphism for detection of sex-specific molecular markers in hop (*Humulus lupulus* L.). Euphytica 151(1): 15–21. doi: 10.1007/s10681-005-9020-4

Divashuk MG, Alexandrov OS, Kroupin PY, Karlov GI (2011) Molecular cytogenetic mapping of *Humulus lupulus* sex chromosomes. Cytogenetic and Genome Research 134(3): 213–219. doi: 10.1159/000328831

Grabowska-Joachimiak A, Mosiolek M, Lech A, Goralski G (2011) C-Banding/DAPI and in situ hybridization reflect karyotype structure and sex chromosome differentiation in *Humulus japonicus* Siebold & Zucc. Cytogenetic and Genome Research 132(3): 203–211. doi: 10.1159/000321584

Grabowska-Joachimiak A, Sliwinska E, Piguła M, Skomra U, Joachimiak AJ (2006) Genome size in *Humulus lupulus* L. and *H. japonicus* Siebold & Zucc. (*Cannabaceae*). Acta Societatis Botanicorum Poloniae 75(3): 207–214.

Howell EC, Armstrong SJ, Filatov DA (2009) Evolution of neo-sex chromosomes in *Silene diclinis.* Genetics 182(4): 1109–1115. doi: 10.1534/genetics.109.103580

Jakse J, Stajner N, Kozjak P, Cerenak A, Javornik B (2008) Trinucleotide microsatellite repeat is tightly linked to male sex in hop (*Humulus lupulus* L.). Molecular Breeding 21(2): 139–148. doi: 10.1007/s11032-007-9114-x

Karlov GI, Danilova TV, Horlemann C, Weber G (2003) Molecular cytogenetic in hop (*Humulus lupulus* L.) and identification of sex chromosomes by DAPI-banding. Euphytica 132(2): 185–190. doi: 10.1023/A:1024646818324

Kazama Y, Sugiyama R, Matsunaga S, Shibata F, Uchida Y, Hizume M (2003) Organization of the *Kpn*I family of chromosomal distal-end satellite DNA in *Silene latifolia.* Journal of Plant Research 116(4): 317–326. doi: 10.1007/s10265-003-0106-6

Kim SY, Kim CS, Lee J, Bang JW (2008) Karyotype analysis and physical mapping using two rRNA genes in dioecious plant, *Humulus japonicus* Siebold & Zucc. Genes Genomics 30(2): 243–251.

Koo DH, Nam YW, Choi D, Bang JW, Jong H, Hur Y (2010) Molecular cytogenetic mapping of *Cucumis sativus* and *C.melo* using highly repetitive DNA sequences. Chromosome Research 18(3): 325–336. doi: 10.1007/s10577-010-9116-0

Kubis S, Schmidt T, Heslop-Harrison JSP (1998) Repetitive DNA elements as a major component of plant genomes. Annals of Botany 82(Supplement A): 45–55. doi: 10.1006/anbo.1998.0779

Laínz M (1963) Aportaciones al conocimiento de la flora cántabro-astur, VII (1). Boletín del Instituto Estudios Asturianos. Suplemento de Ciencias 7: 45.

Linnaeus C (1753) Species plantarum: exhibentes plantas rite cognitas ad genera relatas. Tomus II. Holmiae. Salvii, 1028 pp.

Ming R, Wang J, Moore PH, Paterson AH (2007) Sex chromosomes in flowering plants. American Journal of Botany 94(2): 141–150. doi: 10.3732/ajb.94.2.141

Ming R, Bendahmane A, Renner SS (2011) Sex chromosomes in land plants. Annual Review of Plant Biology 62: 485–514. doi: 10.1146/annurev-arplant-042110-103914

Murakami A, Darby P, Javornik B, Pais MS, Seigner E, Lutz A, Svoboda P (2006) Molecular phylogeny of wild Hops, *Humulus lupulus.* Heredity 97(1): 66–74. doi: 10.1038/sj.hdy.6800839

Ono T (1955) Chromosomes of common hop and its relatives Bulletin of Brewing Science 2: 3–65.

Parker JS, Clark MS (1991) Dosage sex-chromosome systems in plants. Plant Science 80(1-2): 79–92. doi: 10.1016/0168-9452(91)90274-C

Polley A, Seigner E, Ganal MW (1997) Identification of sex in hop (*Humulus lupulus)* using molecular markers. Genome 40(3): 357–361. doi: 10.1139/g97-048

Rogers SO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Molecular Biology Reports 5(2): 69–76. doi: 10.1007/BF00020088

Ruiz Rejón C, Jamilena M, Garrido-Ramos MA, Parker JS, Ruiz Rejón M (1994) Cytogenetic and molecular analysis of the multiple sex chromosome system of *Rumex acetosa.* Heredity 72(2): 209–215. doi: 10.1038/hdy.1994.28

Seefelder S, Ehrmaier H, Schweizer G, Seigner E (2000) Male and female genetic linkage map of hops, *Humulus lupulus.* Plant Breeding 119(3): 249–255. doi: 10.1046/j.1439-0523.2000.00469.x

Siebold PF, Zuccarini JG (1846) Florae Japonicae familae naturales adjectis generum et specierum exemplis selectis. Sectio altera. Plantae dicotyledoneae et monocotyledonae. Abhandelungen der mathematischphysikalischen Classe der Königlich Bayerischen Akademie der Wissenschaften 4(3): 213.

Shephard HL, Parker JS, Darby P, Ainsworth CC (2000) Sexual development and sex chromosomes in hop. New Phytologist 148(3): 397–411. doi: 10.1046/j.1469-8137.2000.00771.x

Winge O (1929) On the nature of sex chromosome in *Humulus.* Hereditas 12(1–2): 53–63. doi: 10.1111/j.1601-5223.1929.tb02497.x

CompCytogen 6(3): 249–266 (2012)
doi: 10.3897/CompCytogen.v6i3.2831
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

A revision of chromosome II (CD) mapping in *Chironomus plumosus* (Linnaeus, 1758) group (Diptera, Chironomidae)

Veronika V. Golygina[1,2], I. I. Kiknadze[2]

1 *Institute of Cytology and Genetics SB RAS*, *Prosp. akademika Lavrentieva 10*, *Novosibirsk 630090*, *Russia*
2 *Novosibirsk State University*, *ul. Pirogova*, *2*, *Novosibirsk*, *630090*, *Russia*

Corresponding author: *VeronikaV. Golygina* (nika@bionet.nsc.ru)

Academic editor: *P. Michailova* | Received 3 February 2012 | Accepted 17 July 2012 | Published 9 August 2012

Citation: Golygina VV, Kiknadze II (2012) A revision of chromosome II (CD) mapping in *Chironomus plumosus* (Linnaeus, 1758) group (Diptera, Chironomidae). Comparative Cytogenetics 6(3): 249–266. doi: 10.3897/CompCytogen.v6i3.2831

Abstract

A revision of the main and alternative banding sequences in chromosome II (CD) has been made for all 14 species of the *Chironomus plumosus* (Linnaeus, 1758) group. A new version of mapping has been suggested for 10 out of 18 banding sequences of arm C and 12 out of 22 banding sequences of arm D. Mapping of 7 banding sequences has been done for the first time according to the Keyl-Devai system. Phylogenetic relationships of banding sequences of chromosome II have been discussed.

Keywords

Chironomus plumosus group, karyotype, banding sequence, chromosome II, mapping

Introduction

The *Chironomus plumosus* (Linnaeus, 1758) group of sibling species is a unique object for the study of patterns in linear reorganization of the genome during speciation, as it consists of species with mainly wide geographic ranges with natural populations at different stages of divergence (Butler et al. 1999; Gunderina et al. 1999a, b; Kiknadze et al. 2000; Golygina et al. 2007). What is more important, the possibility of mapping

all karyotypes in the genus *Chironomus* Meigen, 1803 against one standard species allows us to detect all chromosomal rearrangements that distinguish different species and reconstruct their phylogenetic relationship on the basis of karyological analysis. However, for conducting such a study it is very important to have high resolution photographic maps of karyotypes and a unified mapping system of polytene chromosomes. In our earlier work (Golygina and Kiknadze 2008) we extensively discussed all the difficulties facing a researcher who works with *Ch. plumosus* group and presented a revision of mapping in chromosome I (AB). In this paper we present the results of revision of the main and alternative banding sequences in chromosome II (CD) for 14 *Ch. plumosus* group species.

Methods

Revision of chromosome II (CD) mapping was conducted for 14 *Ch. plumosus* sibling species: *Chironomus agilis* Shobanov et Djomin, 1988, *Ch.* sp. prope *agilis* (working name "*Ch. agilis* 2") (Kiknadze et al. 1991a), *Ch. balatonicus* Devai, Wülker & Scholl, 1983, *Ch. bonus* Shilova & Dzhvarsheishvili, 1974, *Ch. borokensis* Kerkis, Filippova, Shobanov, Gunderina & Kiknadze, 1988, *Ch. entis* Shobanov, 1989, *Ch. muratensis* Ryser, Scholl & Wülker, 1983, *Ch. nudiventris* Ryser, Scholl & Wülker, 1983, *Ch. plumosus* (Linnaeus, 1758), *Ch. sinicus* Kiknadze, Wang, Istomina & Gunderina, 2005, *Chironomus* sp. J (Kiknadze et al., 1991b), *Chironomus* sp. K (Golygina & Ueno, 2005), *Ch. suwai* Golygina & Martin, 2003, *Ch. usenicus* Loginova & Belyanina, 1994. High-resolution photomaps were created for mapping all the banding sequences in question.

Mapping of arms C and D was done according to the Keyl-Devai mapping system (Keyl 1962, Dévai et al. 1989). For banding sequences in arm C of *Ch. balatonicus* the additional letter D was used for designation of regions 23 and 24, i.e. they are designated now as D23 and D24, as these regions originated in arm D and were transferred into arm C as a result of pericentric inversion.

Each banding sequence in each chromosomal arm is given a short designation as followes: three-letter abbreviation of the species name (for example, agi – for *Ch. agilis*, bal – for *Ch. balatonicus*, etc.) is followed by the name of the arm and the serial number of banding sequence in this arm (according to the order of its discovery), and prefixed by a letter that indicates its geographical distribution - p' for Palearctic sequences, n' for Nearctic sequences, or h' for Holarctic sequences (e.g. p'balC1, n'entD4, h'pluD2 etc.).

Equipment in the Centre of Microscopical analysis of biological objects SB RAS in the Institute of Cytology and Genetics (Novosibirsk) was used for this work: microscope "Axioskop" 2 Plus, CCD-camera AxioCam HRc, software package AxioVision 4 (Zeiss, Germany).

Results

Arm C

Mapping of banding sequences of *Ch. plumosus* sibling species according to Keyl-Devai system that was published by now is shown in Table 1. In total 18 banding sequences (14 main and 4 alternative) are considered in this study. A dendrogram of banding sequences constructed on the basis of published mapping is shown in Fig. 1a, where main banding sequences are written in bold and alternative banding sequences in italics. As can be seen, most of the banding sequences of different species were considered to be derivatives from h'pluC2 and its homologous banding sequences: eight blocks of sequences could be derived independently from h'pluC2 by one or more inversion steps.

According to our analysis, 11 banding sequences that belong to 8 species require a revision of mapping in this arm.

In our opinion, the most important changes should be made in mapping of banding sequences of *Chironomus* sp. prope *agilis*, *Ch. balatonicus*, *Ch. muratensis*, *Ch. nudiventris*, and *Chironomus* sp. J, as we suggest a different way of their origination. Banding sequences of *Ch. entis*, *Ch. sinicus* and *Ch. usenicus* required only minor corrections in the mapping of inversion breakpoints.

The banding sequences of *Ch. agilis*, *Ch. bonus*, *Ch. borokensis*, *Ch. plumosus*, *Ch. suwai* and *Chironomus* sp. K remain unchanged (Table 2, Fig. 2a, e, f).

1. Revision of arm C mapping of *Chironomus* sp. prope *agilis* (*Ch. agilis* 2)

According to the previous mapping agi2C1 differs from agiC1 by two non-overlapping paracentric inversions (Kiknadze et al. 1991a). However, our analysis had shown that these two inversions are, in fact, overlapping and slightly bigger than was presumed previously. Due to these rearrangements the region 5b-4h- was transferred to the distal part of the arm, very close to the telomere, whereas bands 2c-1f could be found between regions 6f-c and 5c-f (Fig. 2b, 3a, Table 2).

2. Revision of arm C mapping of *Ch. balatonicus*

Ch. balatonicus differs from all other species of *Ch. plumosus* group by the presence of a complex pericentric inversion in chromosome CD. It was presumed previously (Kiknadze et al. 1996a) that the banding sequence in the centromeric region of *Ch. balatonicus* was formed by four inversions (Table 1). However, comparison of p'balC1 with p'agiC1 and p'nudC1, which have the most clear banding structure in the centromeric region, allowed us to conclude that banding sequence in the centromeric regions of *Ch. balatonicus* differs from other species by three inversions (Fig. 2c, 3c, d, Table 2).

Table 1. Mapping of arm C main and alternative banding sequences in *Ch. plumosus* group before the revision. † – main banding sequences are marked by *, ‡ – papers with given version of the mapping are shown in parenthesis, § – mapping of this banding sequence is given with the same designations as in original paper, i.e. brackets indicate bands from arm D that were transferred into arm C as a result of pericentric inversion.

Designation of banding sequence	Mapping of banding sequence
h'agiC1*†	1a-2c 6c-f 7a-d 16a-17a 6hg 11d-15e 8a-11c 6b-2d 17b-22g C (Kiknadze et al. 1996b, 2004) ‡
p'agi2C1*	1a-2c 17a-16a 7d-a 6f-c 5a-6b 11c-8a 15e-11d 6gh 4i-2d 17b-22g C (Kiknadze et al. 2004)
p'balC1*	1a-2d 6c-e 7a-d 16a-17a 6h-f 11e-12d 4a-6b 11d-8a 15e-13a 3c-2e 17b-22g [24a-e 23a-c 23g-d 24fg] C§ (Kiknadze et al. 1996a)
p'balC2	not mapped in Keyl-Devai system
p'bonC1*	1a-2c 6c-f 7a-d 16a-17a 6hg 11d-12d 4a-6b 11c-8a 15e-13a 3c-2d 17b-22g C (Kiknadze et al. 2004)
p'borC1*	1a-2c 6c-f 7a-d 16a-17a 6hg 11d-12d 4a-6b 11c-8a 15e-13a 3c-2d 17b-22g C (Kiknadze et al. 2004)
p'entC1	1a 14a-11d 6gh 17a-16a 7a-d 6f-c 2c-1b 14b-15e 8a-11c 6b-2d 17b-22g C (Kiknadze et al. 2000)
p'entC2*	1a 11h-d 6gh 17a-16a 7a-d 6f-c 2c-1b 12a-15e 8a-11c 6b-2d 17b-22g C (Golygina 1999, Kiknadze et al. 2000, 2004)
n'entC3	1a 11h-d 6gh 17a-16a 7a-d 6f-c 2c 5a-6b 11c-8a 15e-12a 1b-2b 4i-2d 17b-22g C (Golygina 1999, Kiknadze et al. 2000)
p'murC1*	1a-2c 15e-a 8a-11c 6b-4a 6c-f 7a-d 16a-17a 6gh 11d-12d 14e-13a 3c-2d 17b-22g C (Kiknadze et al. 2004)
p'nudC1*	1a-2c 11d-15e 8a-11c 6b-2d 6c-f 7a-d 16a-17a 6gh 17b-22g C (Kiknadze et al. 2004)
p'pluC1*	1a-2c 6c-f 7a-d 16a-17a 6hg 11d-12d 4a-6b 11c-8a 15e-13a 3c-2d 17b-22g C (Butler et al. 1999, Golygina 1999, Golygina and Kiknadze 2001, Kiknadze et al. 2004)
h'pluC2	1a-2c 6c-f 7a-d 16a-17a 6hg 11d-15e 8a-11c 6b-2d 17b-22g C (Butler et al. 1999, Golygina 1999, Golygina and Kiknadze 2001)
p'sinC1*	1a-c 12d-11d 6gh 17a-16a 7d-a 6f-c 2c-1d 13a-15e 8a-11c 6b-2d 17b-22g C (Kiknadze et al. 2005)
p'spJC1*	not mapped in Keyl-Devai system
p'spKC1*	1a-2c 6c-f 7a-d 5c-6b 11c-8a 15e-11d 6gh 17a-16a 5b-2d 17b-22 C (Golygina and Ueno 2008)
h'suwC1*	1a-2c 6c-f 7a-d 16a-17a 6hg 11d-12d 4a-6b 11c-8a 15e-13a 3c-2d 17b-22g C (Golygina et al. 2003, Kiknadze et al. 2004)
p'useC1*	not mapped in Keyl-Devai system

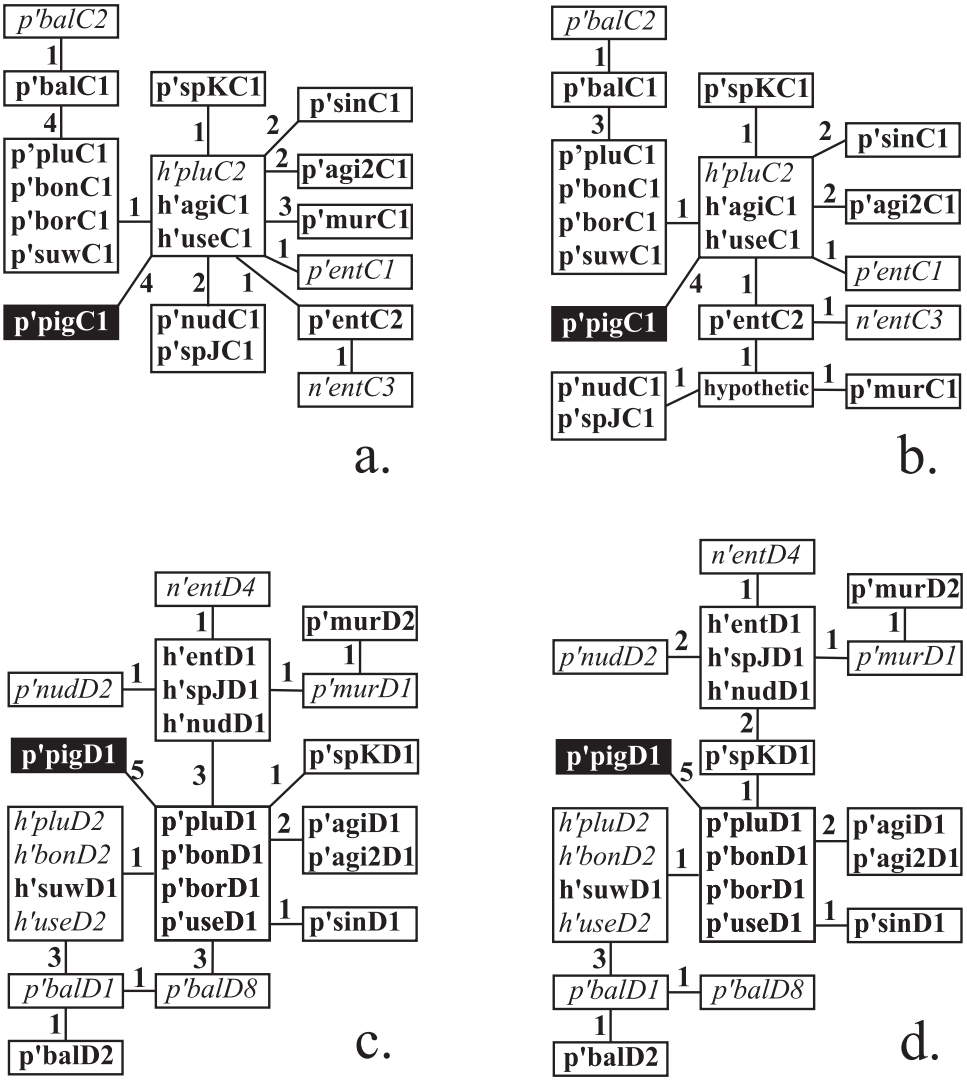


Figure 1. Phylogenetic relationship of main and alternative banding sequences in arms C and D before (**a**, **c**) and after (**b**, **d**) the revision. p'pluC1, h'entD1, n'entD4 etc. are the names of banding sequences considered in this study (please see 'Methods' for the rules of banding sequence designations). Main banding sequences are written in bold, alternative – in italic. Identical banding sequences enclosed in boxes, figures near the lines that connect boxes indicate numbers of inversion steps between banding sequences. The banding sequences p'pigC1 and p'pigD1 belong to *Chironomus piger* and are included into the dendrogramms as an outgroup.

Table 2. Mapping of arm C main and alternative banding sequences in *Ch. plumosus* group after the revision. † – main banding sequences are marked by *, ‡ – parts of the sequences highlighted in bold indicate regions which mapping had been changed as a result of the revision, § – for banding sequences in arm C of *Ch. balatonicus* additional letter D was used for designation of regions 23 and 24, i.e. they are designated now as D23 and D24, as these regions were initially originated in arm D and were transferred into arm C as a result of pericentric inversion. Moreover, regions that are affected by the pericentric inversion are given in the italic.

Designation of banding sequence	Mapping of banding sequence
h'agiC1*†	=h'pluC2
p'agi2C1*	**1a-e 5b-4h** 16h-a 7d-a 6f-c **2c-1f 5c**-6b 11c-8a 15e-11d 6gh **17a 4g**-2d 17b-22g C ‡
p'balC1*	1a-2c 6c-f 7a-d 16a-17a 6hg 11d-12d 4a-6b 11c-8a 15e-13a 3c-2d 17b-22g ***D24c-e D23ba D24b-D23c*** *D24fg C* §
p'balC2	**1a-2c 6c-f 7a-c 15e 8a-11c 6b-4a 12d-11d 6gh 17a-16a 7d 15d-13a 3c-2d 17b-22g *D24c-e D23ba D24b-D23c D24fg C***
p'bonC1*	=p'pluC1
p'borC1*	=p'pluC1
p'entC1	**1a-e** 14a-11d 6gh 17a-16a 7a-d 6f-c 2c-**1f** 14b-15e 8a-11c 6b-2d 17b-22g C
p'entC2*	**1a-d 11f**-d 6gh 17a-16a 7a-d 6f-c 2c-**1e 11g**-15e 8a-11c 6b-2d 17b-22g C
n'entC3	**1a-d 11f**-d 6gh 17a-16a 7a-d 6f-c 2c 5a-6b 11c-8a 15e-**11g 1e**-2b 4i-2d 17b-22g C
p'murC1*	**1a-d 11f-d 6gh 13f-15e** 8a-11c 6b-3c 6c-f 7a-d 16a-17a **13e-11g 1e-2c 3b**-2d 17b-22g C
p'nudC1*	**1a-d 11f-d 6gh 17a 2f-3b 2c-1e 11g**-15e 8a-11c 6b-**3c** 6c-f 7a-d 16a-h **2ed** 17b-22g C
p'pluC1*	1a-2c 6c-f 7a-d 16a-17a 6hg 11d-12d 4a-6b 11c-8a 15e-13a 3c-2d 17b-22g C
h'pluC2	1a-2c 6c-f 7a-d 16a-17a 6hg 11d-15e 8a-11c 6b-2d 17b-22g C
p'sinC1*	1a-**d** 12d-11d 6gh 17a-16a 7d-a 6f-c 2c-**1e** 13a-15e 8a-11c 6b-2d 17b-22g C
p'spJC1*	=p'nudC1
p'spKC1*	1a-2c 6c-f 7a-c 5c-6b 11c-8a 15e-11d 6gh 17a-16a 7d 5b-2d 17b-22g C
h'suwC1*	=p'pluC1
p'useC1*	**1a-2c 6c-f 7a-d 16a-17a 6hg 11d-15e 8a-11c 6b-2d 17b-22g C**

The banding sequence p'balC2 was previously mapped according to Maximova's system only. It originated from p'balC1 by one simple inversion and its up to date mapping in the Keyl-Devai system is shown in Fig. 2d.

3. Revision of arm C mapping of *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, and *Chironomus* sp. J

It was presumed earlier that the main banding sequences of *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris* originated from h'pluC2 independently, and that p'spJC1 of *Chironomus* sp. J is identical to p'nudC1 (Fig. 1a). However, our analysis had shown that whereas this conclusion is true for p'entC1 and p'entC2, main banding sequences of *Ch. muratensis* and *Ch. nudiventris* (and, therefore, *Chironomus* sp. J, where the main banding sequence is indeed identical to p'nudC1) originated from p'entC2

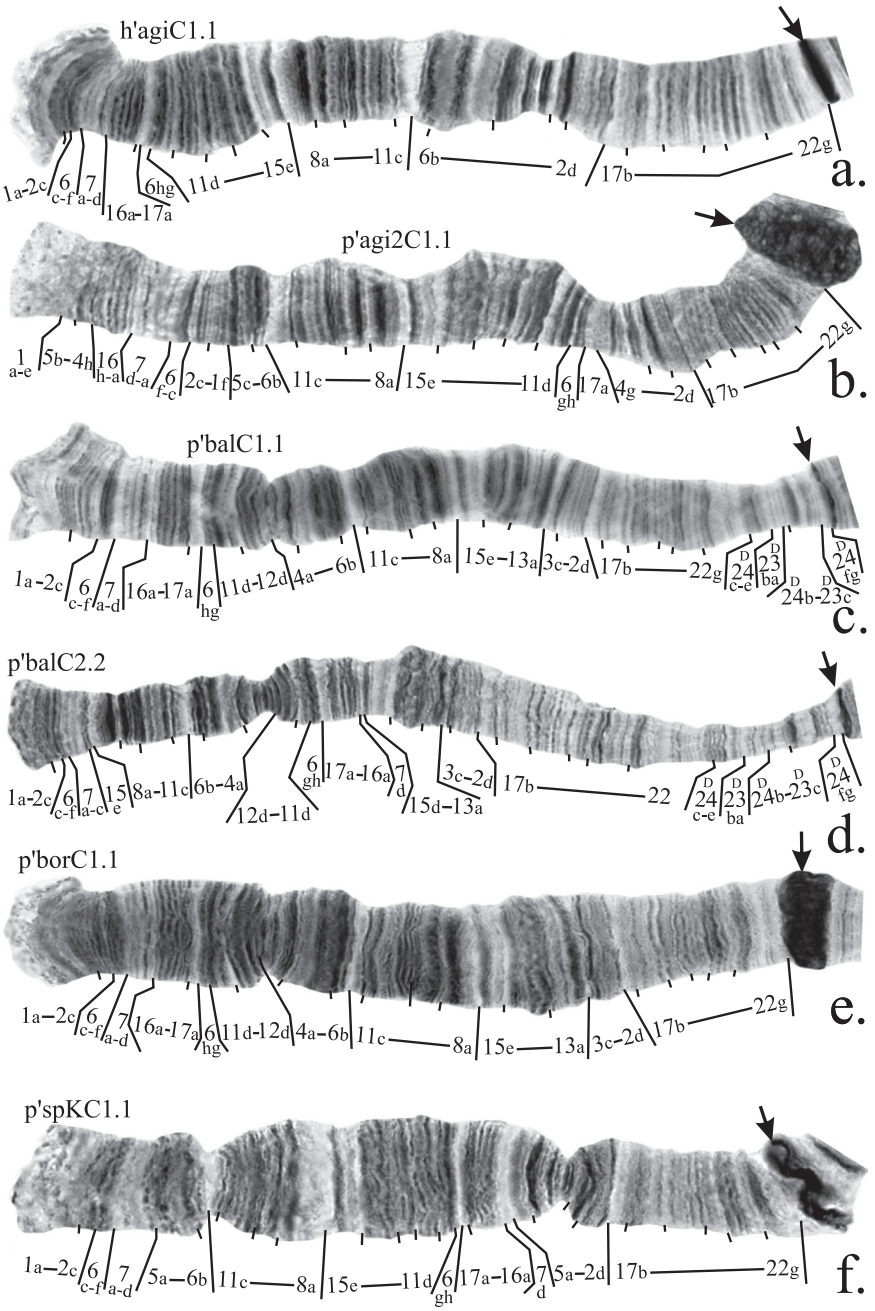


Figure 2a–f. Mapping of banding sequences of *Ch. plumosus* sibling species in arm C. **a** h'agiC1.1 **b** p'agi2C1.1 **c** p'balC1.1 **d** p'balC2.2 **e** p'borC1.1 **f** p'spKC1.1. For banding sequences p'balC1.1 and p'balC2.2 letter D was used for designation of regions 23 and 24, i.e. they are designated now as D23 and D24, as these regions were initially originated in arm D and were transferred into arm C as a result of pericentric inversion. Centromeric bands designated by arrows.

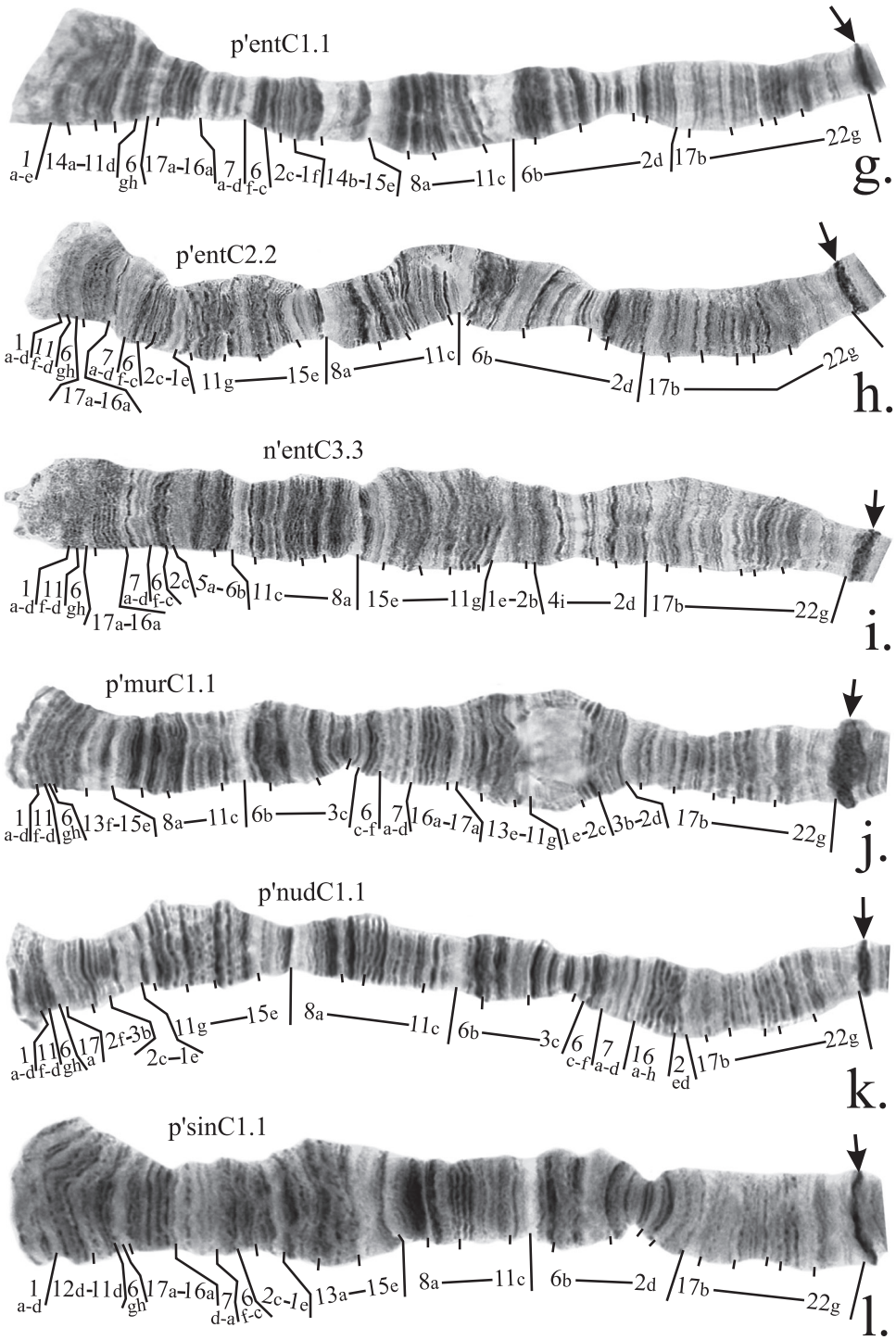


Figure 2g–l. Mapping of banding sequences of *Ch. plumosus* sibling species in arm C (*continued*). **g** p'entC1.1 **h** p'entC2.2 **i** n'entC3.3 **j** p'murC1.1 **k** p'nudC1.1 **l** p'sinC1.1

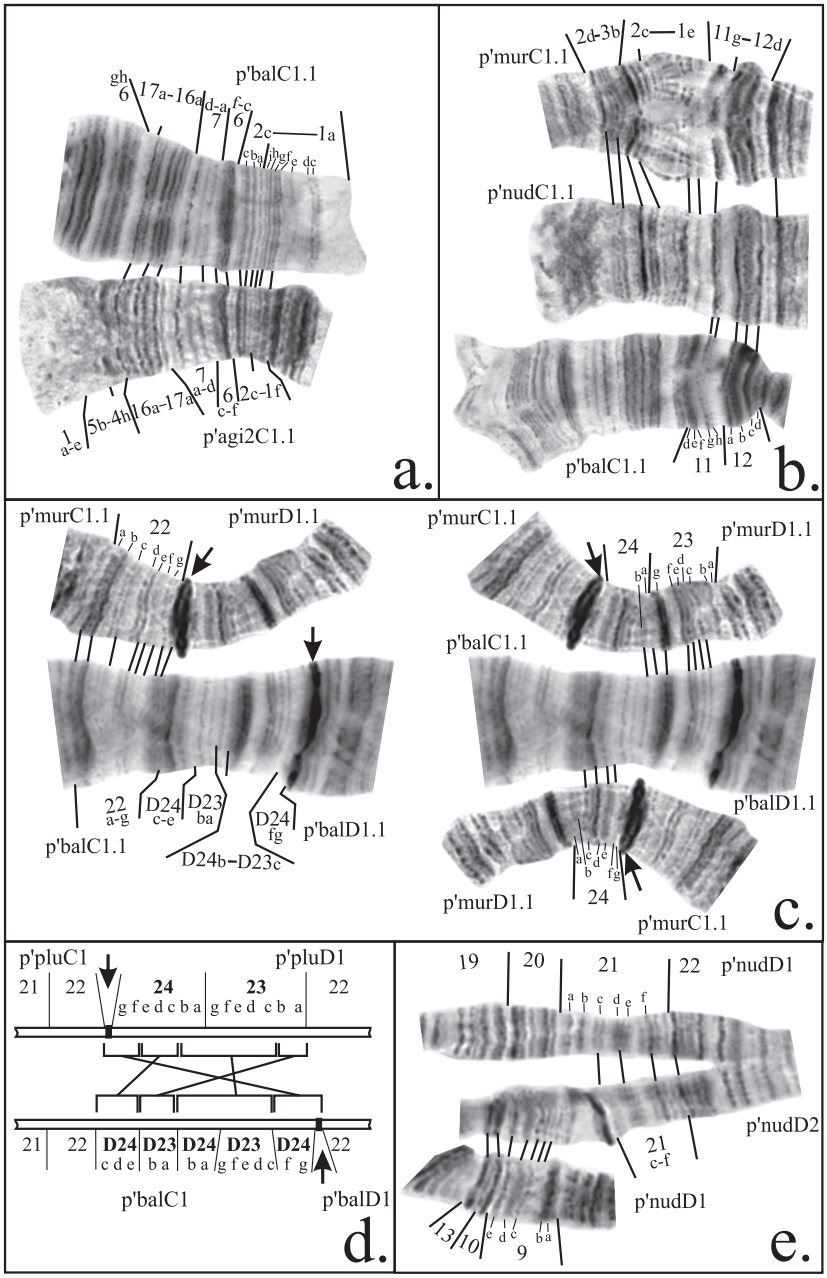


Figure 3a–e. Mapping of some inversion breakpoints in species of *Ch. plumosus* group in arms C and D. **a** comparison of parts of banding sequences p'balC1 and p'agi2C1 **b** comparison of parts of banding sequences p'murC1, p'nudC1 and p'balC1 **c** comparison of parts of banding sequence p'balD1 with p'murD1 and p'murC1 **d** schematic comparison of centromeric regions of chromosome CD of *Ch. plumosus* and *Ch. balatonicus* showing the structure of pericentric inversion in *Ch. balatonicus*, brackets connected by lines indicate groups of bands affected by the inversion **e** comparison of parts of banding sequences p'nudD1 and p'nudD2. Abbreviations are as in Figure 2.

through the same hypothetical banding sequence that at present does not occur in the banding sequence pools of these species (Fig. 1 b). Moreover, as the chromosome banding structure of *Ch. muratensis* and *Ch. nudiventris* is better than of *Ch. entis*, comparison of their banding sequences with h'pluC2 also allowed us to correct mapping of breakpoints of p'entC2.

Ch. entis has three banding sequences that have been found in the homozygous state and, therefore, are considered in this study: p'entC1, p'entC2 and n'entC3. Banding sequence p'entC1 differ from h'pluC2 by a simple inversion. A correction in the mapping of p'entC1 should be made for the left breakpoint of the inversion (Fig. 2g, Table 2).

As was mentioned above, the mapping of banding sequence p'entC2 is crucial for the mapping of n'entC3 and all banding sequences of *Ch. muratensis*, *Ch. nudiventris* and *Chironomus* sp. J. It differs from h'pluC2 by a simple inversion in the distal part of the arm with its left breakpoint located very close to the telomere. Analysis of these regions in the banding sequences of *Ch. muratensis*, *Ch. nudiventris* and *Chironomus* sp. J allowed us to conclude that the real breakpoints of the inversion that distinguish p'entC2 from h'pluC2 fall between bands 1d and 1e on the left border, and 11f and 11h on the right border of the inversion (Fig. 2h, 3b, Table 2).

The mapping of n'entC3 should be corrected in accordance with mapping of p'entC1 (Fig. 2i, Table 2).

Thorough analysis of the main banding sequences of *Ch. muratensis* and *Ch. nudiventris* allowed us to conclude that they originated from p'entC2 through the hypothetical banding sequence:

1a-d 11f-d 6gh 17a-16a 7a-d 6f-c 2c 3c-6b 11c-8a 15e-11g 1e-2b 3b-2d 17b-22g C

Underline indicates simple inversion that distinguishes this banding sequence from p'entC2.

Both p'murC1 and p'nudC1 differ from this hypothetical banding sequence by simple inversions. Their revised mapping is shown in Table 2 and on Fig. 2j, k.

4. Revision of arm C mapping of *Ch. sinicus* and *Ch. usenicus*

Mapping of p'sinC1 require only a minor revision. According to the previous version of the mapping, region 1 was divided by an inversion between bands 1c and 1d. However, we had not been able to locate band 1d near band 13a so we suggest that the left breakpoint of this inversion situated between band 1d and 1e (Fig. 2l, Table 2).

Until now the banding sequence h'useC1 has been mapped only partially (Loginova and Belyanina 1994), although it was indicated that it is identical to h'pluC2. We concur with this statement. Complete mapping of the h'useC1 in Keyl-Devai system is shown in Table 2.

Phylogenetic relationships of revised banding sequences in arm C of the *Ch. plumosus* group species are shown in Fig. 1b.

Arm D

Mapping for banding sequences in this arm that has been published so far is shown in Table 3. Phylogenetic relationship of banding sequences based on this mapping is shown in Fig. 1c. In total 22 banding sequences (14 main and 8 alternative) are considered in this study. Analysis of main and alternative banding sequences in this arm has shown that only minor changes in the mapping of inversion breakpoints are required for some banding sequences. The corrections in mapping should be made for nine banding sequences, belonging to *Ch. agilis*, *Chironomus* sp. prope *agilis*, *Ch. balatonicus*, *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris* and *Chironomus* sp. J. In addition, two banding sequences belonging to *Ch. muratensis* and *Ch. nudiventris* were mapped in Keyl-Devai system for the first time.

Mapping of banding sequences of *Ch. bonus*, *Ch. borokensis*, *Ch. plumosus*, *Ch. sinicus*, *Chironomus* sp. K, *Ch. suwai*, and *Ch. usenicus* remains unchanged (Fig. 4e, f, l, m, Table 4).

1. Revision of arm D mapping of *Ch. agilis* and *Chironomus* sp. prope *agilis*

The banding sequences in arm D of both species are identical. They differ from p'pluC1 by two non-overlapping inversions. A correction should be made for breakpoints of the inversion in the proximal part of the arm: the left breakpoint falls between bands 14a and 14b instead of 14f and 14g, whereas the right breakpoint falls between regions 20 and 21 instead of bands 20b and 20c (Fig. 4a, Table 4).

2. Revision of arm D mapping of *Ch. balatonicus*

As was mentioned previously, *Ch. balatonicus* differs from all other species of *Ch. plumosus* group by the presence of a pericentric inversion in chromosome CD. Due to this, the arm D of *Ch. balatonicus* is shorter than normal and consists of only 22 regions instead of 24.

Ch. balatonicus has three banding sequences that could be found in homozygous state and, therefore, are considered in this study: p'balD1, p' balD2 and p'balD8. Among them p'balD2 and p'balD8 require a minor revision. Banding sequence p'balD2 differ from p'balD1 by simple inversion, according to the previous mapping its right breakpoint was placed between regions 15 and 16, however it is clear that band 15e is not affected by the inversion so the real breakpoint falls between bands 15d and 15e (Fig. 4c, Table 4).

The banding sequence p'balD8 was considered previously as identical to p'pluD1 for all the arm length except the part affected by the pericentric inversion. However, our analysis has shown that this is not the case and p'pluD8 in fact originated from p'balD1 by a simple inversion. As a result, region 11 was broken into two parts and band 11a stayed between regions 10e-b and 13b-17f (Fig. 4d, Table 4).

Table 3. Mapping of arm D main and alternative banding sequences in *Ch. plumosus* group before the revision. † – main banding sequences are marked by *, ‡ – papers with given version of the mapping are shown in parenthesis.

Designation of banding sequence	Mapping of banding sequence
p'agiD1*†	1a-d 4a-7g 18a-d 8a-10a 13a-11a 3g-1e 10e-b 13b-14f 20b-18e 17f-14g 20c-24g C (Kiknadze et al. 2004) ‡
p'agi2D1*	1a-d 4a-7g 18a-d 8a-10a 13a-11a 3g-1e 10e-b 13b-14f 20b-18e 17f-14g 20c-24g C (Kiknadze et al. 2004)
p'balD1	1a-3g 10b-e 4a-7g 18a-d 8a-10a 13a-11a 13b-17f 18e-22e C (Kiknadze et al. 1996a)
p'balD2*	1a-3g 10b-e 4a-7g 18a-d 8a-9e 15e-13b 11a-13a 10a 16a-17f 18e-22e C (Golygina et al. 1996)
p'balD8	1a-3g 11a-13a 10a-8a 18d-a 7g-4a 10e-b 13b-17f 18e-22e C (Golygina et al. 1996)
p'bonD1*	1a-3g 11a-13a 10a-8a 18d-a 7g-4a 10e-b 13b-17f 18e-24g C (Kiknadze et al. 2004)
p'borD1*	1a-3g 11a-13a 10a-8a 18d-a 7g-4a 10e-b 13b-17f 18e-24g C (Kiknadze et al. 2004)
h'borD2	=h'pluD2 (Kerkis et al. 1988), not mapped according to Keyl-Devai system
h'entD1*	1a-2d 15e-16c 18d 8a-10a 13a-12a 18c-a 7g-4a 10e-b 13b-15d 2e-3g 11a-c 16d-17f 18e-24g C (Golygina 1999; Kiknadze et al. 2000)
n'entD4	1a-2d 15e-16c 18d 8a-d 19h-18e 17f-16d 11c-a 3g-2e 15d-13b 10b-e 4a-7g 18a-c 12a-13a 10a-9a 20a-24g C (Golygina 1999)
p'murD1	1a-i 11c-a 3g-2e 15d-13b 10b-e 4a-7g 18a-c 12a-13a 10a-8a 18d 16c-15e 2d-a 16d-17f 18e-24g C (Kiknadze et al. 2004)
p'murD2*	not mapped according to Keyl-Devai system
h'nudD1*	1a-2d 15e-16c 18d 8a-10a 13a-12a 18c-a 7g-4a 10e-b 13b-15d 2e-3g 11a-c 16d-17f 18e-24g C (Kiknadze et al. 2004)
p'nudD2	not mapped according to Keyl-Devai system
p'pluD1*	1a-3g 11a-13a 10a-8a 18d-a 7g-4a 10e-b 13b-17f 18e-24g C (Butler et al. 1999, Golygina 1999, Golygina and Kiknadze 2001, Kiknadze et al. 2004)
h'pluD2	1a-3g 10b-e 4a-7g 18a-d 8a-10a 13a-11a 13b-17f 18e-24g C (Butler et al. 1999, Golygina 1999, Golygina and Kiknadze 2001)
p'sinD1*	1a-2g 13a 10a-8a 18d-a 7g-4a 10e-b 13b-14h 3g-2h 12d-11a 15a-17f 18e-24g C (Kiknadze et al. 2005)
h'spJD1*	not mapped according to Keyl-Devai system
p'spKD1*	1a-3g 11a-13a 10a-8a 16d-13b 10b-e 4a-7g 18a-d 16e-17f 18e-24 C (Golygina and Ueno 2008)
h'suwD1*	1a-3g 10b-e 4a-7g 18a-d 8a-10a 13a-11a 13b-17f 18e-24g C (Golygina et al. 2003, Kiknadze et al. 2004)
p'useD1*	1a-3g 11a-13part 9a-e 18part 8d-4a 10ba 13part-17f 18part-24g C (Loginova and Beljanina 1994)
h'useD2	=h'pluD2 (Loginova and Beljanina 1994), not mapped according to Keyl-Devai system

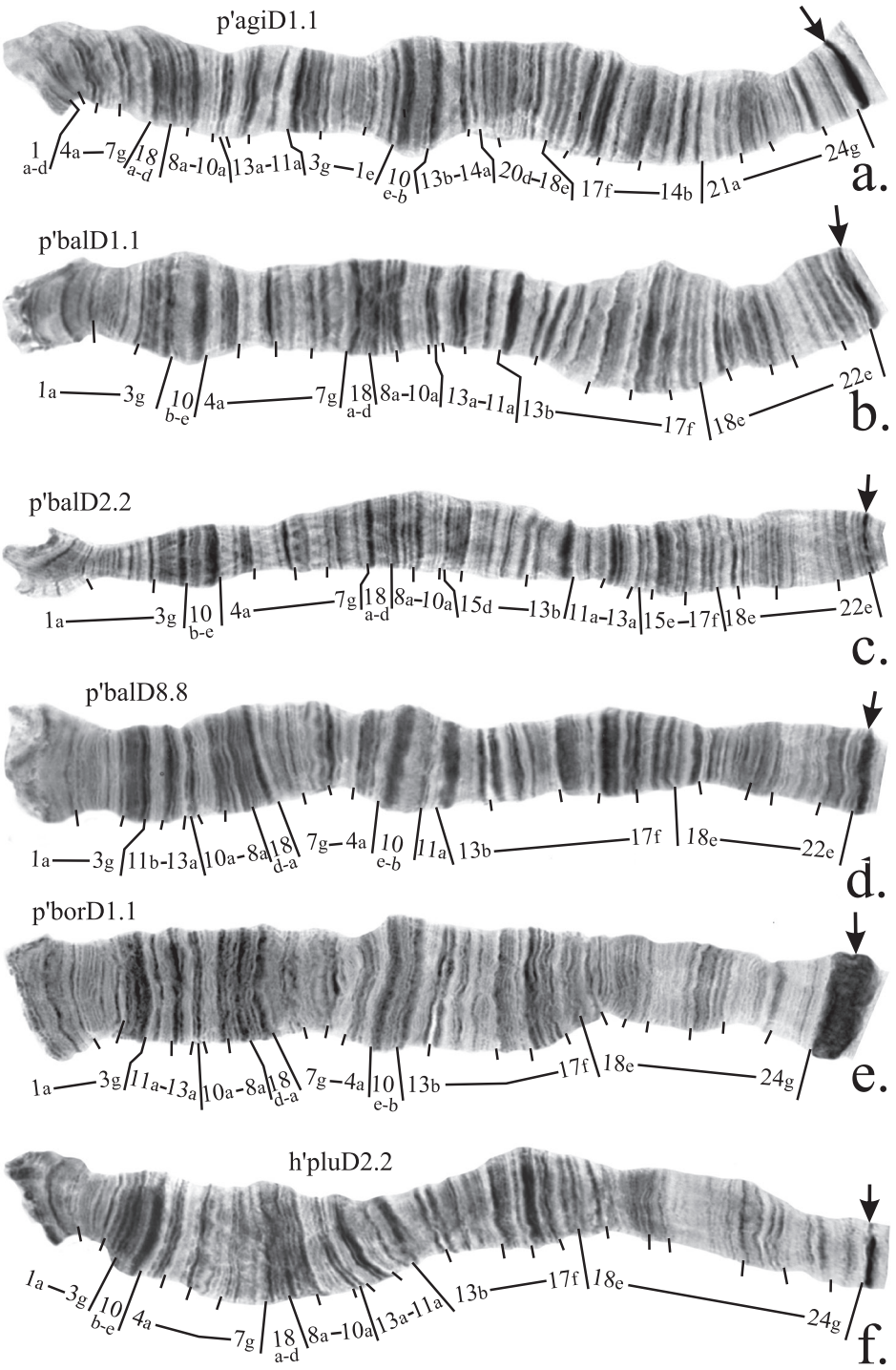


Figure 4a–f. Mapping of banding sequences of *Ch. plumosus* sibling species in arm D. **a** p'agiD1.1 **b** p'balD1.1 **c** p'balD2.2 **d** p'balD8.8 **e** p'borD1.1 **f** h'pluD2.2. Centromeric bands designated by arrows.

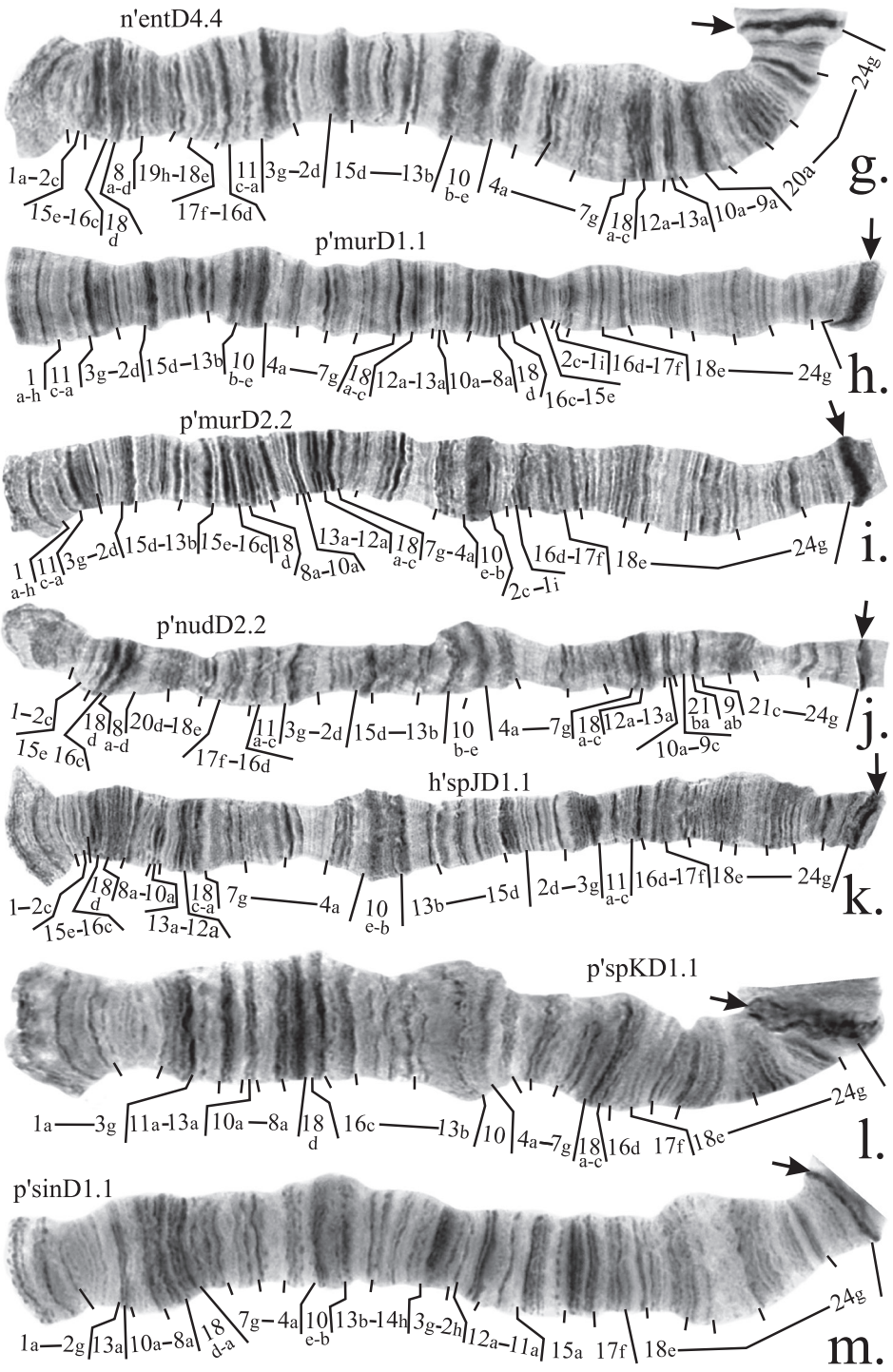


Figure 4g–m. Mapping of banding sequences of *Ch. plumosus* sibling species in arm D (*continued*). **g** n'entD4.4 **h** p'murD1.1 **i** p'murD2.2 **j** p'nudD2.2 **k** h'spJD1.1 **l** p'spKD1.1 **m** p'sinD1.1.

Table 4. Mapping of arm D main and alternative banding sequences in *Ch. plumosus* group after the revision. † – main banding sequences are marked by *, ‡ – parts of the sequences highlighted in bold indicate regions which mapping had been changed as a result of the revision.

Designation of banding sequence	Mapping of banding sequence
p'agiD1*†	1a-d 4a-7g 18a-d 8a-10a 13a-11a 3g-1e 10e-b 13b-**14a 20d**-18e 17f-**14b 21a**-24g C ‡
p'agi2D1*	=p'agiD1
p'balD1	1a-3g 10b-e 4a-7g 18a-d 8a-10a 13a-11a 13b-17f 18e-22e C
p'balD2*	1a-3g 10b-e 4a-7g 18a-d 8a-**10a 15d**-13b 11a-13a **15e**-17f 18e-22e C
p'balD8	1a-3g **11b**-13a 10a-8a 18d-a 7g-4a 10e-b **11a** 13b-17f 18e-22e C
p'bonD1*	=p'pluD1
p'borD1*	=p'pluD1
h'borD2	=h'pluD2
h'entD1*	1a-**2c** 15e-16c 18d 8a-10a 13a-12a 18c-a 7g-4a 10e-b 13b-15d **2d**-3g 11a-c 16d-17f 18e-24g C
n'entD4	1a-**2c** 15e-16c 18d 8a-d 19h-18e 17f-16d 11c-a 3g-**2d** 15d-13b 10b-e 4a-7g 18a-c 12a-13a 10a-9a 20a-24g C
p'murD1	**1a-h** 11c-a 3g-**2d** 15d-13b 10b-e 4a-7g 18a-c 12a-13a 10a-8a 18d 16c-15e **2c-1i** 16d-17f 18e-24g C
p'murD2*	**1a-h 11c-a 3g-2d 15d-13b 15e-16c 18d 8a-10a 13a-12a 18c-a 7g-4a 10e-b 2c-1i 16d-17f 18e-24g C**
h'nudD1*	=h'entD1
p'nudD2	**1a-2c 15e-16c 18d 8a-d 20d-18e 17f-16d 11c-a 3g-2d 15d-13b 10b-e 4a-7g 18a-c 12a-13a 10a-9c 21ba 9ab 21c-24g C**
p'pluD1*	1a-3g 11a-13a 10a-8a 18d-a 7g-4a 10e-b 13b-17f 18e-24g C
h'pluD2	1a-3g 10b-e 4a-7g 18a-d 8a-10a 13a-11a 13b-17f 18e-24g C
p'sinD1*	1a-2g 13a 10a-8a 18d-a 7g-4a 10e-b 13b-14h 3g-2h 12d-11a 15a-17f 18e-24g C
h'spJD1*	=h'entD1
p'spKD1*	1a-3g 11a-13a 10a-8a 18d 16c-13b 10b-e 4a-7g 18a-c 16d-17f 18e-24g C
h'suwD1*	1a-3g 10b-e 4a-7g 18a-d 8a-10a 13a-11a 13b-17f 18e-24g C
p'useD1*	=p'pluD1
h'useD2	=h'pluD2

3. Revision of arm D mapping of *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, *Chironomus* sp. J, and *Chironomus* sp. K

On the basis of our study we suggest that all banding sequences of these species have a common origin (Fig.1d). We believe that the banding sequence p'spKD1 forms the basis of all other banding sequences of these species. It differs from p'pluD1 by a simple inversion (Fig. 4l, Table 4). The main banding patterns of *Ch. entis*, *Ch*, *nudiventris* and *Chironomus* sp. J are identical and originated from p'spKD1 by two inversion steps, correction was made for mapping of region 2: we believe that only bands 2a-c remain at the distal part of the arm whereas band 2d is affected by the inversion (Fig. 4k, Table 4). As n'entD4, p'nudD2, p'murD1 and p'murD2 are derivatives of h'entD1 and its homologous banding sequences, mapping of region 2 in them was also changed.

Thorough analysis of p'nudD2 allowed us to conclude that it differs from h'nudD1, not by one, but by two inversions (Fig. 3e, 4j, Table 4). A small change was also made to the mapping of region 1 of p'murD1: according to previous mapping the left breakpoint was situated between regions 1 and 2, but our analysis had shown that the actual breakpoint falls between bands 1h and 1i (Fig. 4h, Table 4). Mapping of p'murD2 in the Keyl-Devai system is presented for the first time (Fig. 4i, Table 4).

Phylogenetic relationships of the revised banding sequences in arm D of the *Ch. plumosus* group species are shown in Fig. 1d.

Discussion

The revision of banding sequences in chromosome CD of *Ch. plumosus* sibling species has shown that the phylogenetic relationships of banding sequences in both arms are more complex than appeared previously. The changes are not as significant as were made for arm A, for example (Golygina and Kiknadze 2008), but they still affect phylogenetic relationships of banding sequences of four species in arm C and six species in arm D (Fig. 1).

We have shown that banding sequences of *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, and *Chironomus* sp. J in arm C are more closely related than was considered previously and that p'entC2 of *Ch. entis* can be considered as ancestral for banding sequences of other three species.

The most ancient banding sequence in arm C should be considered h'pluC2 and the identical banding sequences of *Ch. agilis* and *Ch. usenicus* as they are the closest to the p'pigC1.

In general, analysis of the phylogeny of banding sequences in arm C has shown that this arm has the highest level of divergence in comparison to arms A, B, and D, as only three clusters of homologous banding sequences exist in this arm, whereas there are four such clusters in arm A and D and seven in arm B (Fig. 1b, Golygina and Kiknadze 2008). Moreover, six species have species specific main and alternative banding sequences (Fig. 1b).

The revision in arm D mostly provided minor changes in the mapping of inversion breakpoints without affecting phylogenetic relationships of banding sequences in general. The only significant change has come from the correction of the inversion breakpoint of p'spKD1 which has made it the ancestor for all banding sequences of *Ch. entis*, *Ch. muratensis*, *Ch. nudiventris*, *and Chironomus* sp J. In general, the banding sequences in arm D show a significant level of divergence with four species that have species specific main and alternative banding sequences and several complex inversions that distinguish banding sequences from one another.

Considering the high level of banding sequence divergence in both arms, it can be stated that chromosome CD is the most divergent among the three big chromosomes of *Chironomus* karyotype and probably plays a more important role in speciation than the other two.

Acknowledgements

Financial support was provided by grant RFBR 09-04-01440 and grants from the Presidium of Russian Academy of Sciences: "Biodiversity and dynamics of gene pools" and "Origin and evolution of biosphere".

References

Butler MG, Kiknadze II, Golygina VV, Martin J, Istomina AG, Wülker WF, Sublette JE, Sublette MF (1999) Cytogenetic differentiation between Palearctic and Nearctic populations of *Chironomus plumosus* L. (Diptera, Chironomidae). Genome 42(5): 797–815.

Dévai Gy, Miskolczi M, Wülker W (1989) Standartization of chromosome arms B, C and D in *Chironomus* (Diptera: Chironomidae). Acta biologica Debrecina : supplementum Oecologica Hungarica 2(1): 79–92.

Golygina VV (1999) Divergence of karyotypes of Holarctic *Chironomus* species from *Ch. plumosus* group in Paleartic and Nearctic (Diptera, Chironomidae). Ph.D. Dissertation, Novosibirsk, Russian Federation: Institute of Cytology and Genetics, Russian Academy of Sciences, 132 pp. [In Russian]

Golygina VV, Istomina AG, Rakisheva AZh, Kiknadze II (1996) New banding sequences in the *Chironomus balatonicus* karyofund (banding sequence pool). Tsitologiya 38(8): 869–883. [In Russian]

Golygina VV, Kiknadze II (2001) Karyofund (banding species pool) of *Chironomus plumosus* (Diptera, Chironomidae) in Palearctic. Tsitologiya 43(5): 507–519. [In Russian]

Golygina VV, Martin J, Kiknadze II, Siirin M, Ivanchenko OV, Makarchenko EA (2003) *Chironomus suwai*, a new species of the *plumosus* group (Diptera, Chironomidae) from Japan. Aquatic Insects 25(3): 177–189. doi: 10.1076/aqin.25.3.177.15258

Golygina VV, Ueno R (2008) Chironomus sp. K – a new member of plumosus sibling-group from Japan. Contemporary Aquatic Entomological Study in East Asia - Proceedings of 3rd International Symposium on Aquatic Entomology in East Asia, Tianjin, China. Nankai University Press, 32–39.

Golygina VV, Kiknadze II (2008) The revision of chromosome I (AB) mapping in *Chironomus plumosus* group (Diptera: CHironomidae). Comparative Cytogenetics 2(1): 37–55.

Gunderina LI, Kiknadze II, Golygina VV (1999a) Differentiation of the cytogenetic structure of natural populations in the *plumosus* group of sibling species *Chironomus balatonicus*, *Chironomus entis*, *Chironomus muratensis*, and *Chironomus nudiventris* (Chironomidae: Diptera). Russian Journal of Genetics 35(5): 506–513.

Gunderina LI, Kiknadze II, Golygina VV (1999b) Intraspecific differentiation of the cytogenetic structure in natural populations of *Chironomus plumosus* L., the central species in the group of sibling species (Chironomidae: Diptera). Russian Journal of Genetics 35(2): 142–150.

Keyl H-G (1962) Chromosomenevolution bei *Chironomus*. II. Chromosomenumbauten und phylogenetische Beziehungen der Arten. Chromosoma 13(4): 464–514. doi: 10.1007/BF00327342

Kiknadze II, Siirin MT, Filippova MA, Gunderina LI, Kalachikov SM (1991a) The change of the pericentromeric heterochromatin mass is one of important ways of the chironomid evolution. Tsitologiya 33(12): 90–98. [In Russian]

Kiknadze II, Shilova AI, Kerkis IE, Shobanov NA, Zelentzov NI, Grebenjuk LP, Istomina AG, Prasolov VA (1991b) Karyotypes and morphology of larvae in the tribe *Chironomini*. Novosibirsk, 113 pp. [In Russian]

Kiknadze II, Golygina VV, Istomina AG (1996a) The mapping of chromosomal arms C and D in the midge *Chironomus balatonicus.* Tsitologiya 38(7): 674–680. [In Russian]

Kiknadze II, Istomina AG, Gunderina LI, Aimanova KG, Salova TA, Savvinov DD (1996b) Chironomid karyofunds (banding species pools) of Yakutian cryolitozone (permafrost). Novosibirsk, 166 pp. [In Russian]

Kiknadze II, Butler MG, Golygina VV, Martin J, Wülker WF, Sublette JE, Sublette MF (2000) Intercontinental karyotypic differentiation of *Chironomus entis* Shobanov, a Holarctic member of the *C. plumosus* group (Diptera, Chironomidae). Genome 43(5): 857–873.

Kiknadze II, Golygina VV, Istomina AG, Gunderina LI (2004) Pattern of chromosomal polymorphism during population and species divergence in *Chironomus* (Diptera, Chironomidae). Sibirskiy Ekologicheskiy Zhurnal 11(5): 635–652. [In Russian]

Kiknadze II, Wang X, Istomina AG, Gunderina LI (2005) A new *Chironomus* species of the *plumosus*-group (Diptera, Chironomidae) from China. Aquatic Insects 27(3): 199–211. doi: 10.1080/01650420500101770

Loginova NV, Belyanina SI (1994) A new species of the genus *Chironomus* from the group *plumosus - Chironomus usenicus* sp. n. (Diptera, Chironomidae). Zoologicheskiy Zhurnal 73(11): 93–105. [In Russian]

CompCytogen 6(3): 267–271 (2012)
doi: 10.3897/CompCytogen.v6i3.3019
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

A comparison of the chromosome G-banding pattern in two *Sorex* species, *S. satunini* and *S. araneus* (Mammalia, Insectivora)

Yuriy M. Borisov[1], Victor N. Orlov[1]

1 *Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Leninskii pr., Moscow, 119071 Russia*

Corresponding author: *Yuriy M. Borisov* (boris@sevin.ru)

Academic editor: *V. Lukhtanov* | Received 27 February 2012 | Accepted 19 June 2012 | Published 20 August 2012

Citation: Borisov YM, Orlov VN (2012) A comparison of the chromosome G-banding pattern in two *Sorex* species, *S. satunini* and *S. araneus* (Mammalia, Insectivora). Comparative Cytogenetics 6(3): 267–271. doi: 10.3897/CompCytogen.v6i3.3019

Abstract

The G-banded karyotype of *S. satunini* was compared with the karyotype of *Sorex araneus*. Extensive homology was revealed. The major chromosomal rearrangements involved in the evolutionary divergence of these species have been identified as centric fusions and centromeric shifts. From the known palaeontological age of *S. satunini* it is obvious that the vast chromosomal polymorphism of the *S. araneus* group originated during the middle Pleistocene.

Keywords

G-banding, common shrew, *Sorex araneus, Sorex satunini,* karyotype, phylogeny

Introduction

Within the genus *Sorex* Linnaeus, 1758, the *Sorex araneus* group includes eight species characterized by the sex chromosome complex XY_1Y_2 (Zima et al. 1998). Some of them were raised to species status on a karyological basis. Two species from this group, the common shrew *Sorex araneus* Linnaeus, 1758 and the Caucasian shrew *S. satunini* Ognev, 1922 can be defined as cryptic species that are virtually impossible to distinguish by morphological (cranial) characters (Sokolov and Tembotov 1989).

The common shrew is widely distributed in Europe and Asia up to as far east as Lake Baikal, and the Caucasian shrew is known to be present in the Caucasus and in

the northern parts of Asia Minor (Sokolov and Tembotov 1989, Bukhnikashvili and Kryštufek 2008). In the North Caucasian plains, this species is contiguous with the common shrew (the chromosomal race Neroosa) (Stacheev et al. 2010).

The common shrew displays phenomenal variability of the autosomal complement (Wójcik et al. 2002). The Caucasian shrew is monomorphic and can be reliably identified by means of conventionally stained karyotype (Kozlovsky 1973, Sokolov and Tembotov 1989, Macholán 1996). Macholán (1996) recognized in G-banded metaphases of the *S. satunini* the autosomes *af, bc* and *tu,* which are invariantly present in the common shrew karyotype. The presence of these Robertsonian fusions in the *S. satunini* corroborates the findings of Zagorodniuk and Khazan (1996) who described the arm combinations of autosomes *af, bc, gh, ik, jn, lo*, and *tu* in the karyotype of a single female from Kobi (Georgia).

From the plain between the Kuban and Don rivers we described a new subspecies of the Caucasian shrew *Sorex satunini tembotovi* Orlov, Balakirev, Borisov, 2010 (Orlov et al. 2010) that differs from the subspecies *S. s. armenica* Sokolov et Tembotov, 1989 and *S. s. stavropolica* Sokolov et Tembotov, 1989.

In this study the karyotypes of *S. s. tembotovi* and *S. araneus* (chromosome race Moscow) were examined and compared.

Material and methods

Three females and four males of *S. satunini* were captured in the valley of the Beisoog River (45º40'N, 39º41'E), 90 km N of the Krasnodar city in June 2009. Two shrews of the race Moscow (male and female) were captured in Moscow vicinity.

Mitotic chromosome spreads were prepared in the field conditions from bone marrow and spleen cells using the air-drying technique after fixation with methanol and glacial acetic acid. For G-banding, the slides were treated with trypsin solution according to Seabright (1971). Chromosome nomenclature used follows Searle et al. (2010).

Results and comments

The karyotype of *S. satunini* consists of 24–25 chromosomes. The sex chromosomes are a large metacentric X, a small acrocentric Y_1, and a medium-sized Y_2. Of 11 autosomal pairs, only a single pair of small chromosomes is acrocentric, all other autosomes are biarmed. Such a karyotype has been described for many populations from the North Caucasian and Transcaucasian regions (Kozlovsky 1973, Sokolov and Tembotov 1989, Macholán 1996).

The Caucasian shrew has the following chromosome formula: XX / XY_1Y_2, *af, bc, gh, ik, jn, lo, tu, m, p, q, r, tu*. The comparison of the G-banded metaphase chromosomes of *S. satunini* and of *S. araneus* (the race Moscow) is presented in Fig. 1. This comparison revealed a considerable homology between individual chromosomal arms. Identical

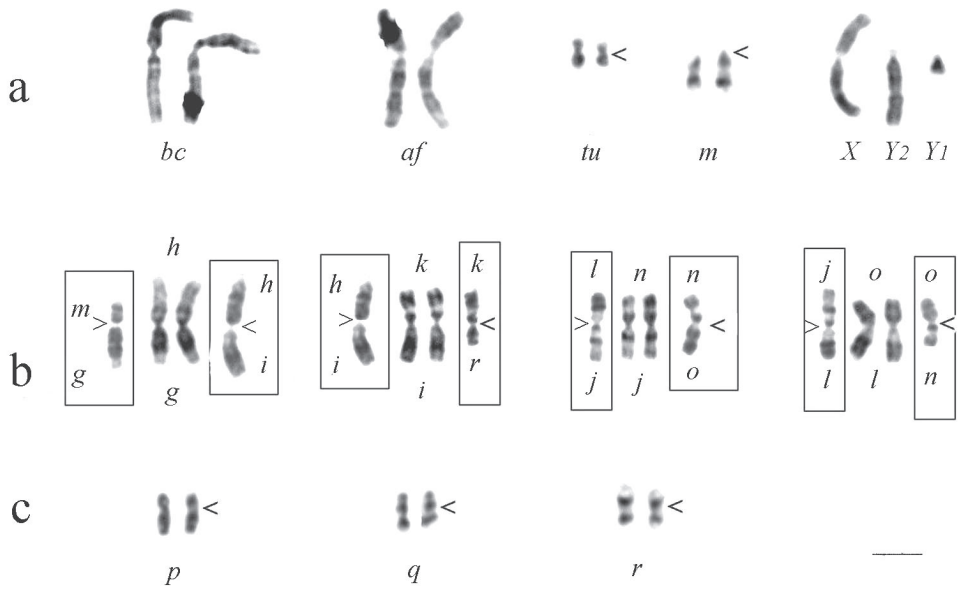


Figure 1. The G-banded karyotype of *Sorex satunini* (male) in comparison with the karyotype of *S. araneus* (the race Moscow, male). The chromosomes of the race Moscow are given in the frameworks. Some chromosomes are identical (**a**), the others are different because of the arms involved in different fusions (**b**) or because of the centromeric shift (**c**). Centromere position is indicated by "<". Bar = 3 μm.

banding patterns and centromeric positions were found in two large biarmed autosomes *af* and *bc*, in small metacentric *tu*, in acrocentric pare *m* and in the sex chromosomes (Fig. 1a). The acrocentric *m* is found in the karyotypes of the chromosomal races of *S. araneus* either as an individual acrocentric, or in combination with other acrocentrics.

Seven arms of *S. araneus*, namely *g, i, k, j, n, l,* and *o,* were also identified in the complement of *S. satunini*. The difference in G-banding of the arm *h* between *S. satunini* and other species of the *S. araneus* group was observed (Fig. 1b). An identical banding pattern and a different centromeric position were found in three autosomal pairs: *p, q*, and *r*, suggesting occurrence of centromeric shift. The chromosomes *p, q,* and *r* were found to be metacentric in the complement of *S. satunini* (Fig. 1c) and acrocentric in *Sorex araneus.*

The large biarmed chromosome *bc* was also identified in the complement of *S. antinorii* Bonaparte, 1840, and only in *S. coronatus* Millet, 1828 it was substituted by *ci* (Hausser and Jammot 1974). The biarmed chromosome *lo* is found in the karyotypes of *S. coronatus* and *S. antinorii,* the biarmed chromosome *jn* - in the karyotype of *S. coronatus* (Brünner et al. 2002). The metacentric *ik* is known in five chromosomal races of *S. araneus* (Wójcik et al. 2003). The metacentric *gh* was identified only in the complement of *S. satunini* (Fig. 1b).

In karyotype of *S. coronatus* there are only two species-specific chromosome rearrangements (Rb fusions *ci* and *mp*). In karyotype of *S. antinorii* there are only two spe-

cies-specific chromosome rearrangements, too (*hj* and *kn*). In karyotype of *S. satunini* there are five species-specific chromosome rearrangements (Rb fusion *gh*, centromeric shifts in the chromosomes *p, q, r,* and, probably, a paracentric inversion in the chromosomal arm *h*), i.e. *S. satunini* has more rearranged karyotype than the species of *S. araneus* group in Western Europe.

A number of chromosome rearrangements shared by *S. araneus, S. satunini* and *S. antinorii* (centric fusions *bc*), by *S. coronatus, S. satunini* and *S. antinorii* (*lo*), by *S. coronatus* and *S. satunini* (*jn*) suggest the existence of a common ancestral species in the Pleistocene of Europe analogous to the modern *S. araneus*.

The known paleontological age points to an early origin of *S. satunini.* At present, the dating of fossils confirmed by the radiocarbohydrate analysis is known only for *S. satunini.* These fossils, morphologically very similar to the recent *S. satunini* were found in the Transcaucasian region (Kudaro caves) in all layers of the middle and late Pleistocene, beginning since 0.36 Myr BP (Osipova 2006).

Acknowledgments

We are grateful to V.V. Stacheev, A.E. Balakirev for the assistance during the expedition and E.V. Cherepanova for the comments on the manuscript. The study was supported by the Russian Foundation for Basic Research (grant 12-04-00551) and Program of the Russian Academy of Sciences “Dynamics and conservation of gene pools”.

References

Brünner H, Lugon-Moulin N, Balloux F, Fumagalli L, Hausser J (2002) A taxonomical reevaluation of the chromosome race Valais of the common shrew, *Sorex araneus* (Insectivora: Soricidae), from multiple, independent characters. Acta Theriologica 47: 245–275.

Bukhnikashvili A, Kryštufek B (2008) *Sorex satunini* IUCN 2011. IUCN Red List of Threatened Species. Version 2011.1. www.iucnredlist.org

Hausser J, Jammot D (1974) Etude biométrique des mâchoires chez les *Sorex* du groupe *araneus* en Europe continentale (Mammalia, Insectivora). Mammalia 38: 324–343. doi: 10.1515/mamm.1974.38.2.324

Kozlovsky AI (1973) Somatic chromosomes of two species of the shrews in Caucasus. Zoologicheski Zhurnal 52: 571–576 [in Russian].

Macholán M (1996) Chromosomal and allozyme characterization of the Caucasian shrew, *Sorex satunini*, from north-eastern Turkey. Hereditas 125: 225–231.

Orlov VN, Balakirev AE, Borisov YuM (2010) A new subspecies of the Caucasian shrew *Sorex satunini* (Mammalia) and phylogenetic relationships of the species inferred from mtDNA sequences and chromosomal markers. Journal of Ecology 1: 111–114. [in Russian].

Osipova VA (2006) Fossil history of shrews (Family Soricidae) in Caucasus. Ph.D. Dissertation, St. Peterburg, Russian Federation: Zoological Institute, Russian Academy of Sciences. [in Russian]

Seabright MA (1971) A rapid banding technique for human chromosomes. Lancet 2 (7731): 971–972. doi: 10.1016/S0140-6736(71)90287-X

Searle JB, Fedyk S, Fredga K, Hausser J, Volobouev VT (2010) Nomenclature for the chromosomes of the common shrew (*Sorex araneus*). Comparative Cytogenetics 4: 87–96. doi: 10.3897/compcytogen.v4i1.28

Sokolov VE, Tembotov AK (1989) Mammals of the Caucasus. Insectivora. Nauka Press, Moscow, 548 pp. [in Russian]

Stacheev VV, Balakirev AE, Grigoryeva OO, Shestak AG, Potapov SG, Borisov YuM, Orlov VN (2010) Distribution of cryptic shrew species of the genus *Sorex* (Mammalia) on the plain between the Don and Kuban rivers with molecular marker diagnostics. Povolzhskiy Journal of Ecology 4: 396–403. [in Russian]

Wójcik JM, Borodin PM, Fedyk S, Fredga K, Hausser J, Mishta A, Orlov VN, Searle JB, Volobouev VT, Zima J (2003) The list of the chromosome races of the common shrew *Sorex araneus* (updated 2002). Mammalia 68: 169–178.

Wójcik JM, Ratkiewicz M, Searle JB (2002) Evolution of the common shrew Sorex araneus: chromosomal and molecular aspects. In: Gliwicz J (Ed) Theriology at the turn of a new century. Białowieża, Polish Academy of Sciences. Acta Theriologica 47(Suppl. 1): 139–167.

Zagorodniuk I, Khazan M (1996) Cladogenesis in the *Sorex araneus* group and its biogeographic interpretation. Abstracts of the ISACC Fourth International Meeting. Norr Malma and Uppsala, August 22-26, 1996. Uppsala University, Uppsala, 38.

Zima J, Lukáčová L, Macholán M (1998) Chromosomal evolution in shrews. In: Wójcik JM, Wolsan M (Eds) Evolution of Shrews. Mammal Research Institute, Polish Academy of Sciences, Białowieża: 175–218.

CompCytogen 6(3): 273–286 (2012)
doi: 10.3897/CompCytogen.v6i3.3380
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Physical mapping of 5S and 18S-5.8S-26S RNA gene families in polyploid series of *Cenchrus ciliaris* Linnaeus, 1771 (Poaceae)

Amina Kharrat-Souissi[1], Sonja Siljak-Yakovlev[2], Fatima Pustahija[2,3], Mohamed Chaieb[1]

1 *Faculté des Sciences de Sfax, U.R. Diversité végétale & Ecosystèmes en Milieu Aride, B.P. 1171, 3000, Sfax, Tunisie* **2** *Univ. Paris-Sud, Ecologie, Systématique, Evolution, UMR 8079 CNRS- AgroParisTech-UPS, Université Paris-Sud, Bâtiment 360, 91405 Orsay Cedex, France* **3** *Faculty of Forestry, University of Sarajevo, Zagrebacka 20, 71000 Sarajevo, Bosnia and Herzegovina*

Corresponding author: *A. Kharrat-Souissi* (kharratsouissi@yahoo.fr)

Academic editor: *Julio R. Daviña* | Received 12 May 2012 | Accepted 10 July 2012 | Published 20 August 2012

Citation: Kharrat-Souissi A, Siljak-Yakovlev S, Pustahija F, Chaieb M (2012) Physical mapping of 5S and 18S-5.8S-26S RNA gene families in polyploid series of *Cenchrus ciliaris* Linnaeus, 1771 (Poaceae). Comparative Cytogenetics 6(3): 273–286. doi: 10.3897/CompCytogen.v6i3.3380

Abstract

The Buffelgrass (*Cenchrus ciliaris* L., Poaceae) is one of the most important pasturage grasses due to its high productivity and good forage qualities. This species possess a high adaptability to bioclimatic constraints of arid zones and may be used for the restoration of degraded arid ecosystems. Tunisian populations present three ploidy levels (4x, 5x and 6x) with a basic chromosome number x=9. This study reported for the first time the distribution of the ribosomal genes (rRNA) for pentaploid and hexaploid cytotypes of *C. ciliaris*. Molecular cytogenetic study using double fluorescence *in situ* hybridization has shown that the two rDNA families, 5S and 18S-5.8S-26S (18S), displayed intraspecific variation in number of loci among different ploidy levels. Each ploidy level was characterized by specific number of both 5S and 18S rDNA loci (two loci in tetraploid, five in pentaploid and six in hexaploid level). For three studied cytotypes (4x, 5x and 6x) all 5S rDNA loci were localized on the subcentromeric region of chromosomes, while 18S loci were situated on the telomeric region of short chromosome arms. Data of the FISH experiments show proportional increase of ribosomal loci number during polyploidization processes.

Keywords

Buffelgrass, *Cenchrus ciliaris*, fluorescence *in situ* hybridization, fluorochrome banding, polyploidy, rDNA organization

Introduction

In the south of Tunisia, the ecosystems are characterized by a high level of anthropogenic disturbance and have been characterized by several factors such as climatic variations and overgrazing (Le Houérou and Hoste 1977). These ecosystems are subjected to high aridity, decrease of biological productivity (Floret et al. 1981), where the perennial species are most affected (Jauffret and Lavorel 2003). Thanks to its high productivity, good forage qualities, fast growth and spreading (Stieber and Wipff 2003) *Cenchrus ciliaris* (syn. *Pennisetum ciliare* (L.) Link) is one of the most promising grass species for rehabilitation of arid rangelands and erosion control in Tunisia and other arid and semi-arid regions. This species occurs widely in tropical, subtropical and warm temperate regions (Watson and Dallwitz 1992), where it represents the species with high pastoral value (Le Houérou and Ionesco 1973). *Cenchrus ciliaris* is especially important in the semi-arid regions because of its high tolerance and adaptability to hot and dry environments (Hall 2001), and its resistance to cutting (Chaieb et al. 1996). This species is highly polymorphic and variable for several morphological traits of ecological and agronomic importance (Mseddi et al. 2004). The embryological and karyological studies of *C. ciliaris* have shown the aposporous mode of reproduction followed by pseudogamy (Ozias-Akins et al. 2003).

Most flowering plants are polyploids, since polyploidization is a ubiquitous event in plant evolution (Wendel 2000). The widespread occurrence of a polyploidy has been attributed to the potential of polyploid species to adapt to a wide range of habitats and survive better in unstable climates than their diploid progenitors (Heslop-Harrison 2000). *Cenchrus ciliaris* posses three ploidy levels: tetraploid (2n=4x=36), pentaploid (2n=5x=45) and hexaploid (2n=6x=54) (Fisher et al. 1954). Most of natural populations around geographical range of species are tetraploid (Fisher et al. 1954). Recently, in the natural Tunisian populations, all three ploidy levels have been discovered (Kharrat-Souissi et al. in press). Namely, in the panel of Tunisian investigated material, most populations are hexaploid, few of them are pentaploid, and one is tetraploid. The genome size of natural populations of *C. ciliaris* was previously determined by Kharrat-Souissi et al. (in press). It ranged from 2C=3.04 to 4.61 pg, revealing three ploidy levels corresponding to 4x, 5x, 6x, with mean 2C DNA amount of 3.04, 3.77 and 4.48 pg respectively. However the only previous data concerning DNA content of *C. ciliaris* are those of Burson et al. (2002). They analysed the genome size on material resulting from experimental progeny of six *C. ciliaris* populations (tetraploid and pentaploid) which were self-pollinated or/and cross-pollinated with *C. setigerus*.

The cytogenetic information provided by combination of chromosome banding and fluorescence *in situ* hybridization (FISH) can be useful for comparing the populations of the same species (Muratović et al. 2005), species within the same genus (Siljak-Yakovlev et al. 2003, Bogunic et al. 2006, Cabral et al. 2006), as well as species of different genera (Fregonezi et al. 2004). This technique was used for physical mapping of genes, karyotyping and analysis of genome organization (De Jong 2003, Jiang

and Gill 2006). In this study the FISH was used in population study of *C. ciliaris* from different geographic origins (from the north to the south of Tunisia).

The objective of the current study was to elucidate the possible changes in number and location of rDNA sites through different ploidy levels of *C. ciliaris* by physical mapping of 5S and 18S rRNA genes.

Material and methods

Plant material and chromosome preparation

The geographical origins of *C. ciliaris* samples collected in natural populations in Tunisia are given in Table 1 and Fig. 1. The vouchers were deposited at the herbarium of the Laboratory of Plants Diversity and Ecosystems in Arid Areas, Department of Biology, University of Sfax. The seedlings were germinated on moist filter papers in Petri dishes at 28°C. After three days, the root tip meristems were removed from germinated seed-

Table 1. Geographical origin, genome size, ploidy level, number of 5S and 18S rDNA loci in Tunisian populations of *Cenchrus ciliaris.* † Data from Kharrat-Souissi et al. (in press); SD, standard deviation.

Localities	Population Code	Latitude	Longitude	2C DNA in pg†	2n†	Ploidy level (x)†	Number of 5S rDNA signals	Number of 18S rDNA signals
South of Tunisia city	MR01	36°73' N	10°24'E	3.03 ±0.03SD	36	4	4	4
East of Teboulta	SA02	35°56'N	11°06'E	4.56±0.01	54	6	6	6
Meknassi Pist	ME04	34°32'N	10°06'E	3.74±0.09	45	5	5	5
Haddej Pist I	ME06	34°26'N	09°12'E	4.34±0.06	54	6	6	6
Haddej pist II	ME08	34°24'N	09°29'E	3.63±0.03	45	5	5	5
Gabès	ME09	34°10'N	09°59'E	4.55±0.03	54	6	6	6
El Hamma - Menzel Habib	ME10	34°02'N	09°44'E	4.46±0.09	54	6	6	6
Gabès- 45 Km – Medenin	JF12	33°37'N	10°28'E	4.57±0.05	54	6	6	6
Metameur-18Km-Toujane	JF14	33°24'N	10°16'E	4.49±0.11	54	6	6	6
IRA of Ben Guerdane-35 km-sidi Mahdi	ST24	32°49'N	11°20'E	4.47±0.04	54	6	6	6
National park of Sidi Toui (Est)	ST25	32°43'N	11°14'E	4.48±0.04	54	6	6	6
IRA of Ben Guerdane - 50 km - Sidi Mahdi	ST26	32°42'N	11°18'E	4.30±0.08	54	6	6	6
Remada-Dhibat (oued el Anguar)	DH28	32°08'N	10°32'E	4.34±0.04	54	6	6	6

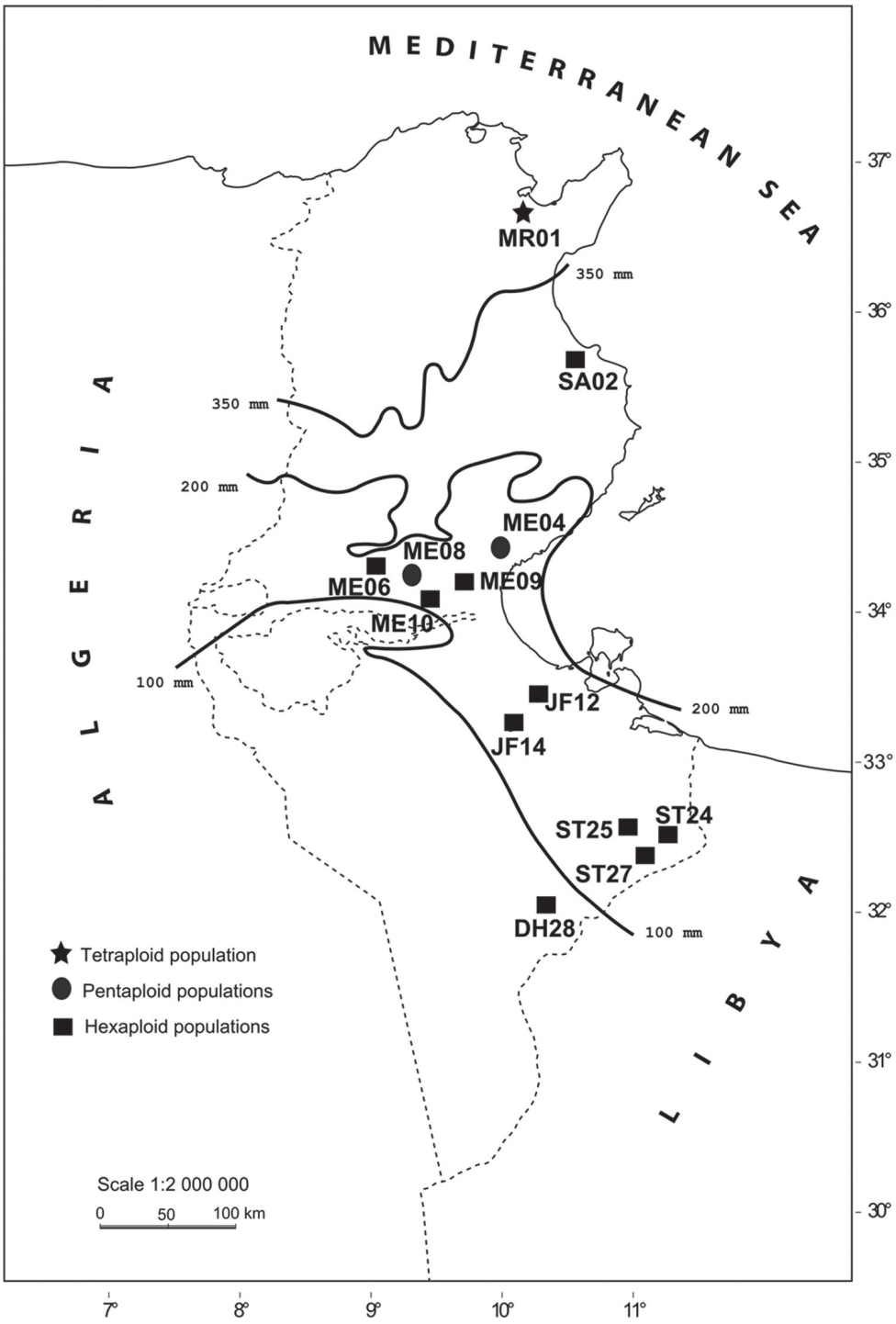


Figure 1. Geographical origin of 13 populations of *Cenchrus ciliaris* in Tunisia.

lings and treated with 2 mM 8-hydroxyquinoline solution for 3 h at 16°C. Subsequently, the material was fixed in freshly prepared ethanol: acetic acid (3:1, v/v) solution.

A slightly modified air drying technique (Geber and Schweizer 1987) was used for chromosome preparations. Five root tips were washed in 0.01M citrate buffer (pH 4.6) for 10 min, and removed to the enzyme mixture (4% R-10 cellulase /Yakult Honsha Co. Tokyo, Japan/, 1% pectolyase Y-23 /Seishin Co., Tokyo, Japan/, 4% hemicellulase /Sigma, France/) for approximately 25 min at 37°C, depending on the root size. Macerated meristems were washed with the same buffer and centrifuged 2 times (4.000 rpm, 5 min), and then the cells suspension was fixed in ethanol:acetic acid solution (3:1, v/v) and centrifuged. The final pellet was resuspended in 50 µl of the same fixative solution. Protoplasts were dropped on a clean slide and kept at room temperature for drying.

Fluorochrome banding

GC-rich heterochromatin staining with chromomycin A_3 (CMA_3, Sigma Aldrich Co., Steinheim, Germany) was performed following Schweizer (1976) with minor modification as described by Siljak-Yakovlev et al. (2002). After incubation in McIlvain buffer pH 7 (with 5 mM $MgSO_4$,) during 15 min and staining with CMA_3 (0.2 mg/ml in same buffer) for 7, 30 or 90 min in dark, the slides were rinsed in the same buffer and counterstained with methyl green (0.5 % in McIlvain buffer pH 5.5) for 7 min and finally were rinsed in McIlvaine buffer pH 5.5. The slides were mounted in the Citifluor, AF1 anti-fade agent (Agar Scientific, Stansted Essex, UK).

Fluorescence *in situ* hybridization (FISH)

The FISH experiment was carried out with two different specific probes of ribosomal DNA (rDNA) simultaneously according to the protocol of Heslop-Harrison et al. (1991). One of the probes is a clone of 4 kb EcoRI fragment, including 18S-5.8S-26S rDNA from *Arabidopsis thaliana* Linnaeus Heynh., 1842 labeled with direct Cy3 (Amersham, Saclay, France). The second probe was the pTa 794 clone containing 410 bp BamHI fragment of the 5S rDNA from wheat labeled with Digoxigenin-11-dUTP (Roche Diagnostics, Meylan, France). Slides were counterstained and mounted in Vectashield medium containing DAPI (4,6-diamidino-2-phenylindole, Vector laboratories, Peterborough, UK) and observed with an epifluorescence Zeiss Axiophot microscope (filter sets 01, 07, 15 and triple 25). The acquisition and treatment of images were performed using a highly sensitive CCD camera (RETIGA 2000R, Princeton Instruments, Evry, France) and an image analyzer (MetaVue, Evry, France). The FISH experiments were carried out for several individuals from one tetraploid population, two pentaploid populations and ten hexpaloid populations.

Karyological analyses

At least five metaphasic chromosome plates were used for karyometrical analysis and construction of idiogram. Chromosomes were classified according to their size and shape related to the centromere position. Terminology used for centromere position follows that of Levan et al. (1964). The total chromosome length for each pair was calculated as the sum of the short and the long arm. Determination of centromere position centromeric index [i%=(short arm/long+short arms)×100] and chromosome type arm ratio (r=long arm/short arm) were performed following nomenclature of Levan et al. (1964).

Results

Distribution of GC rich DNA and constitutive heterochromatin

The chromosomes of *Cenchrus ciliaris* stained with CMA_3 generally showed the pale fluorescent bands of GC rich DNA detected with difficulty after 90 min of staining, and not easily visible on microphotographs (Fig. 2A–B–C). These GC rich bands were occasionally well visible only in hexaploids, where the maximum of three to four signals colocalized with 18S rDNA loci were observed (Fig. 2D). The chromomycin A_3 positive signals were also observed in the interphase nuclei (Fig. 2E) which demonstrated the heterochromatin nature of this GC rich DNA. The clear centromeric $DAPI^+$ bands, corresponding to constitutive heterochromatin, were observed after FISH experiments, but only for tetraploid population (Fig. 2A'–F).

Physical mapping of ribosomal genes

The results of 5S and 18S ribosomal genes mapping in *C. ciliaris* showed that tetraploid population exhibited four signals for both rRNA gene families (Fig. 2A'). In two studied pentaploid populations from different geographical origins (Table 1), five signals of both 5S and 18S rDNA were observed (Fig. 2B'), while six signals were detected in hexaploid populations (Fig. 2C'). It was observed that number of 5S and 18S rDNA loci increased, as expected, with ploidy level (Fig. 2): tetraploid individuals possessed four, pentaploids five, and hexaploids six loci. The 18S rDNA loci had terminal, while 5S rDNA presented pericentromeric localization (Fig. 2). The signals of 5S and 18S rDNA slightly vary in size and intensity, which was probably related to variation in number of copies. The results of CMA staining and FISH experiment on the same metaphase plate show that the GC rich regions, when they are detected as strong bands, correspond to the FISH signals of 18S rDNA probe (Fig. 2D–D').

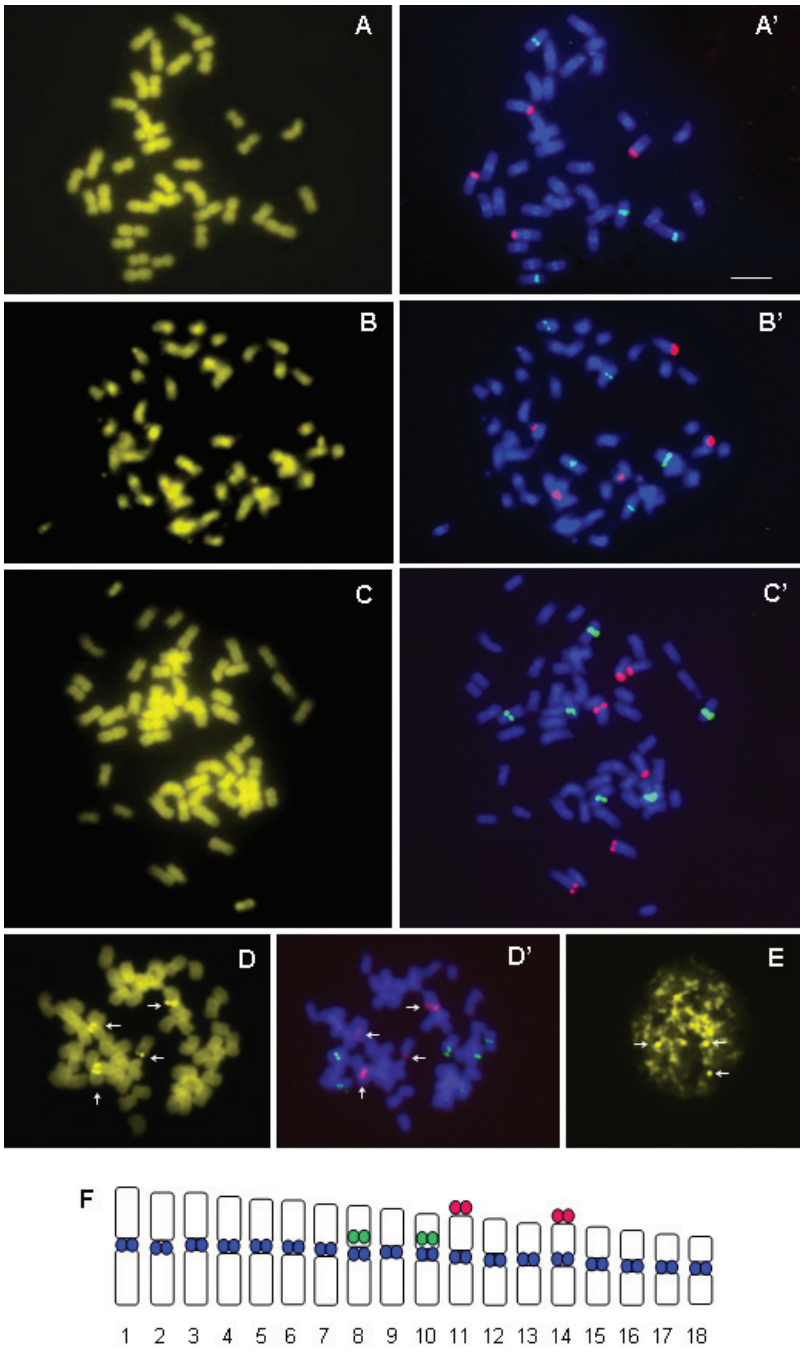


Figure 2. A–F Chromomycin banding and fluorescence *in situ* hybridization of 5S rDNA (green) and 18S rDNA (red) probes respectively on the same chromosome plate: tetraploid individuals (**A**, **A**'); pentaploid individuals (**B**, **B**') and hexaploid individuals (**C**, **C**'); CMA+ signals (**D**) correspond to 18S rDNA loci (**D**') in hexaploid individuals; CMA+ signals in interphase nuclei (**E**); Idiogram of tetraploid individuals, with location of 5S (green), 18S (red) and DAPI (blue) signals (**F**). Bar = 10μm.

Chromosome identification and construction of idiogram

Because many of the chromosomes are similar in size and morphology, chromosome identification for karyotype analysis is very difficult for *C. ciliaris*. Thus, in present study we constructed the idiogram (Fig. 2F) for tetraploids based on conventional morphometry (Table 2) and determined the number and position of rRNA gene loci. The average chromosome length varied from 1.82 to 3.43 µm. The value of R (ratio between the longest and shortest chromosome pair, according to Stebbins 1971) was 0.9, and asymmetry index was 47.89 %. The similarity in chromosome size, difficulties in determining the centromere position and identification of homologous chromosomes makes idiogram construction for pentaploids and hexaploids too difficult.

Table 2. Morphometric data concerning the karyotype of tetraploid *Cenchrus ciliaris* individuals. R = 0.9; AsI % = 47.89; s, short arm; l, long arm; c, total chromosome length; l/s, ratio of long and short arms; i, centromeric index = 100 x s/(l+s); m, metacentric chromosome type (according to Levan et al. 1964); R = the ratio of the longest to the shortest chromosome pair (according to Stebbins 1971); AsI % = (å L / å L+S) x 100 (according to Arano and Saito 1980); SD, standard deviation.

Chromosome pair	s (µm)	l (µm)	c (µm)	(l/s)	i	Chromosome type
1	1.64 (0.15) ^{SD}	1.79 (0.12)	3.43	1.09	47.92	m
2	1.54 (0.18)	1.72 (0.12)	3.27	1.12	47.27	m
3	1.46 (0.20)	1.77 (0.11)	3.23	1.21	45.30	m
4	1.48 (0.19)	1.65 (0.14)	3.13	1.11	47.29	m
5	1.42 (0.17)	1.64 (0.13)	3.06	1.16	46.36	m
6	1.42 (0.17)	1.62 (0.12)	3.04	1.14	46.76	m
7	1.34 (0.17)	1.60 (0.13)	2.94	1.19	45.59	m
8	1.34 (0.18)	1.50 (0.12)	2.84	1.12	47.17	m
9	1.21 (0.17)	1.54 (0.14)	2.75	1.26	44.16	m
10	1.17 (0.16)	1.46 (0.13)	2.63	1.24	44.56	m
11	1.13 (0.18)	1.38 (0.09)	2.51	1.23	44.84	m
12	1.13 (0.11)	1.31 (0.09)	2.44	1.17	46.15	m
13	1.05 (0.12)	1.27 (0.10)	2.32	1.20	45.38	m
14	1.04 (0.11)	1.26 (0.09)	2.29	1.22	45.14	m
15	1.01 (0.13)	1.15 (0.13)	2.16	1.14	46.69	m
16	0.98 (0.13)	1.09 (0.12)	2.07	1.11	47.41	m
17	0.91 (0.15)	1.05 (0.11)	1.96	1.16	46.36	m
18	0.84 (0.10)	0.98 (0.12)	1.82	1.17	46.08	m

Discussion

Heterochromatin pattern

The GC-rich DNA regions detected in hexaploids are distributed in telomeric regions of chromosomes and corresponded to the 18S rDNA loci. This colocalization of GC rich heterochromatin and rDNA has already been reported for numerous

plants and animal species (Siljak-Yakovlev et al. 2003 and references therein, Hamon et al. 2009, Muratović et al. 2010, Bogunic et al. 2011). After prolongation of staining time with CMA_3, signals found in the rare cases were not visible in all 18S rDNA loci. The lack of CMA signals in 18S rDNA sites can be explained by low number of GC pair repetitions (at least four according Godelle et al. 1993). The same phenomenon was observed in *Hydrangea aspera* David Don, 1799-1841 (Mortreau et al. 2010). The centromeric $DAPI^+$ bands were observed only in tetraploid population. DAPI used as a counterstaining in FISH experiments after denaturation/renaturation of DNA reveals heterochromatin as demonstrated by several authors (Siljak-Yakovlev et al. 2002, Muratović et al. 2005, Bogunic et al. 2006 and 2011, Barros e Silva and Guerra 2010, Muratović et al. 2010).

rDNA gene organization

It was obvious to notice that the number of 5S and 18S rDNA sites corresponded to the ploidy level. In tetraploid individuals it was four, in pentaploids five and in hexaploids six signals. Similar phenomenon occurred in polyploids of some other genera, such as *Saccharum* Linnaeus, 1753 (D'Hont et al. 1998), *Passiflora* Linnaeus, 1753 (De Melo and Guerra 2003) and *Ipomoea* Linnaeus, 1753 (Srisuwan et al. 2006).

Tetraploid individuals of *C. ciliaris* show four signals for both 5S and 18S, the same result obtained by Akiyama et al. (2005). Pentaploid and hexaploid cytotypes of *C. ciliaris* have not been previously analysed for their rDNA patterns. Our results show that polyploidy is associated with increase in number of rDNA loci. Similar result was observed by Akiyama et al. (2008) on *Panicum maximum* Jacq, 1786. They found that the numbers of 5S rDNA loci in the diploids and tetraploids were two and four, respectively. Also, the FISH data obtained by Srisuwan et al. (2006) on *Ipomoea* species indicated that the number of 5S rDNA loci corresponded and increased linearly with the ploidy level while the number of 18S rDNA loci decrease in polyploid *I. batatas*. Adachi et al. (1997) found that in polyploid series of *Brochyscome lineariloba* (De Candolle) Druce 1917, the number of 5S rDNA sites increased linearly with the ploidy level, but 18S–26S rDNA was restricted to a single major locus. A proportional gain of ribosomal loci from hexaploid level to octoploid has also been observed in *Artemisia mendozana* De Candolle, 1837 (Pellicer et al. 2010). Loss or addition of rDNA loci during the evolution of a polyploid plant species has been documented in *Triticum* Linnaeus, 1753 (Mukai et al. 1991, Kim et al. 1993), *Gossypium* Linnaeus, 1753 (Crane et al. 1993, Hanson et al. 1996), and *Avena* Linnaeus, 1753 (Jellen et al. 1988).

A comparison of hybridization patterns between the two probes revealed identical results within each ploidy level of *C. ciliaris*. Thus the number of 18S and 5S rDNA loci in different individuals of each ploidy level was constant. This can be explained by the genome stability occurring in the three ploidy levels. This highly conserved nature of both 5S and 18S rDNA loci during polyploid evolution within *C. ciliaris* is not in accordance with observations made in hexaploid of *Ipomea batata*, indeed within this

cytotype some varieties presented 18 signals of 18S and other 12 signals (Srisuwan et al. 2006). Therefore, during polyploid evolution, plant species differ in the degree of the stability of rDNA sites, and different species show different trends in rDNA site-number change. Despite a conserved organization of rDNA sites within each ploidy level, recent molecular investigation of the three ploidy levels of *C. ciliaris* in Tunisia (Kharrat-Souissi et al. 2011) revealed the existence of completely distinct genotypes for pentaploids, suggesting that this cytotype may has two different origins.

In analyzed individuals of *C. ciliaris* all 5S rDNA loci were localized on paracentromeric region of chromosome pairs 8 and 10, while 18S loci were situated on telomeric region of short chromosome arm of pairs 11 and 14. Akiyama et al. (2005) detected the same position for 18S rDNA signals, but they located the 5S signals on the long arm adjacent to the centromere. In our tetraploid population the 5S rDNA sites were observed on the short chromosome arms (Fig. 2F). Chromosome measurements and accurate location of centromere position could explain this difference in the position of 5S signals.

The size and the intensity of both hybridization signals slightly varied among investigated individuals. This can be explained by different copy number of repeats among rDNA sites which has been also detected in several other plant species (Weiss-Schneeweiss et al. 2003, Srisuwan et al. 2006).

The distribution of investigated populations of *C. ciliaris* in Tunisia follows a north-south bioclimatic gradient, where ploidy level and genome size are increasing with aridity (Kharrat-Souissi et al. in press). Namely, tetraploids are present in the most humid areas, pentaploids exist in the center of the country, whilst hexaploids occur from the semi-arid to the Saharan limits, suggesting that this cytotype is better adapted to different environmental conditions (Fig. 1). Hamon et al. (2009) reported that the number of 18S and 5S rDNA appears to be correlated with the genome size and the geographic distribution of the *Coffea* Linnaeus, 1753 species.

In the present study the number of signals of 5S and 18S rDNA loci in pentaploids was intermediate between tetraploids and hexaploids. This result seems to indicate that pentaploid individuals might have derived from hybridization events between tetraploids and hexaploids. Although the apomictic mode of reproduction known as apospory displayed by most *C. ciliaris* genotypes, rare sexual individuals have been identified (Fisher et al. 1954, Sherwood et al. 1980). Using AFLP markers for the Tunisian *C. ciliaris*, differences between individuals descending from the same plant mother were observed. This unexpected level of variability for an apomictic species suggests that the sexual mode of reproduction is not rare in native populations of *C. ciliaris*. The mode of reproduction of the three ploidy levels of *C. ciliaris* in Tunisia was investigated using Flow Cytometric Seed Screening (FCSS; Kharrat-Souissi et al. in press). Observations using FCSS (high 2C embryo peak with a smaller 3C endosperm peak) do not clarify the reproductive mode of the investigated individuals, because the endosperm cells of both the aposporous and sexual plants yield 3C values (Kharrat-Souissi et al. in press). In the case of the aposporous *C. ciliaris*, forming an embryo sac of *Panicum* type, which produces four unreduced nuclei per ovule, the traditional cytological technique of dissecting immature ovaries would appear more appropriate (Visser et al. 2000).

Our data of the FISH experiments show proportional increase of ribosomal loci number during polyploidization processes. However ploidy level increases with aridity (from tetraploid to hexaploid) and give a cytogenetic basis to the considerable differentiation noted between north (humid area) and south (arid area) Tunisian populations of *C. ciliaris*.

Acknowledgments

The authors are grateful to O. Robin for assistance in cytogenetic and to A. Vucicevic for linguistic help. They are also indebted to the Ministry of Education and Science of Tunisia, CNRS (Centre National de la Recherche Scientifique) and Comité Mixte de Coopération Universitaire France-Tunisie (CMCU, project n° 09G 0901) for their financial support.

References

Adachi J, Watnabe K, Fukui K, Ohmido N, Kosuge K (1997) Chromosomal location and reorganization of the 45S and 5S rDNA in the *Brachyscome linearriloba* complex (Asteraceae). Journal Plant Research 110: 371–377. doi: 10.1007/BF02524936

Akiyama Y, Hanna WW, Ozias-Akins P (2005) High-resolution physical mapping reveals that the apospory-specific genomic region (ASGR) in *Cenchrus ciliaris* is located on a heterochromatic and hemizygous region of a single chromosome. Theoretical and Applied Genetics 111: 1042–1051. doi: 10.1007/s00122-005-0020-5

Akiyama Y, Yamada-Akiyama H, Yamanouchi H, Takahara M, Ebina M, Takamizo T, Sugita SI, Nakagawa H (2008) Estimation of genome size and physical mapping of ribosomal DNA in diploid and tetraploid guineagrass (*Panicum maximum* Jacq.). Grassland Science 54: 89–97. doi: 10.1111/j.1744-697X.2008.00110.x

Arano H, Saito H (1980) Cytological studies in family *Umbelliferae* 5. Karyotypes of seven species in subtribe Seselinae. La Kromosomo, 2: 471–480.

Barros e Silva AE, Guerra M (2010) The meaning of DAPI bands observed after C -banding and FISH procedures. Biotechnic and Histochemistry 85: 115–125. doi: 10.3109/10520290903149596

Bogunic F, Muratović E, Siljak-Yakovlev S (2006) Chromosomal differentiation between *Pinus heldreeichii* and *Pinus nigra*. Annals of Forest Science 63: 267–274. doi: 10.1051/forest:2006005

Bogunic F, Siljak-Yakovlev S, Muratović E, Pustahija F, Medjedovic S (2011) Molecular cytogenetics and flow cytometry reveal conserved genome organization in *Pinus mugo* and *P. uncinata.* Annals of Forest Science 68: 179–187. doi: 10.1007/s13595-011-0019-9

Burson BL, Hussey MA, Actkinson JM, Shafer GS (2002) Effect of pollination time on the frequency of $2n + n$ fertilization in apomictic Buffelgrass. Crop Science 42: 1075–1080. doi: 10.2135/cropsci2002.1075

Cabral JS, Felix LP, Guerra M (2006) Heterochromatin diversity and its co-localization with 5S and 45S rDNA sites in chromosomes of four *Maxillaria* species (Orchidaceae). Genetics and Molecular Biology 29: 659–664. doi: 10.1590/S1415-47572006000400015

Chaieb M, Henchi B, Boukhris M (1996) Impact of clipping on root systems of 3 grass species in Tunisia. Journal of Range Management 49: 336–339. doi: 10.2307/4002593

Crane CF, Price HJ, Stelly DM, Czeschin DGJ, Mcknight TD (1993) Identification of homeologous chromosome pairs by *in situ* DNA hybridization to ribosomal RNA loci in meiotic chromosomes of cotton (*Gossypium hirsutum*). Genome 36: 1015–1022. doi: 10.1139/g93-135

D'Hont A, Ison D, Alix K, Roux C, Glaszmann JC (1998) Determination of basic chromosome numbers in the genus *Saccharum* by physical mapping of ribosomal RNA genes. Genome 41: 221–225. http://www.nrcresearchpress.com/doi/pdf/10.1139/g98-023

De Jong H (2003) Visualizing DNA domains and sequences by microscopy: a fifty-year history of molecular cytogenetics. Genome 46: 943–946. doi: 10.1139/G03-107

De Melo NF, Guerra M (2003) Variability of the 5S and 45S rDNA sites in *Passiflora* L. species with distinct base chromosome number. Annals of Botany 92: 309–316. doi: 10.1093/aob/mcg138

Fisher WD, Bashaw EC, Holt EC (1954) Evidence for apomixis in *Pennisetum ciliare* and *Cenchrus setigerus*. Agronomy Journal 46: 401–404. doi: 10.2134/agronj1954.00021962004600090002x

Floret C, Le Floc'h E, Romane F, Pontanier R (1981) Dynamique des systèmes écologiques de la zone aride. Application à l'aménagement sur les bases écologiques d'une zone de la Tunisie présaharienne. Acta Oecologica 2: 195–214. http://www.documentation.ird.fr/hor/fdi:28728

Fregonezi JN, Torezan JMD, Vanzela ALL (2004) A karyotypic study of three southern Brazilian Asteraceae species using fluorescence *in situ* hybridization with a 45S rDNA probe and C-CMA_3 banding. Genetics and Molecular Biology 27: 223–227. doi: 10.1590/S1415-47572004000200016

Geber G, Schweizer D (1987) Cytochemical hetero- chromatin differentiation in *Sinapis alba* (Cruciferae) using a simple air-drying technique for producing chro- mosome spreads. Plant Systematic and Evolution 158: 97–106. doi: 10.1007/BF00936336

Godelle B, Cartier D, Marie D, Brown CS, Siljak-Yakovlev S (1993) Heterochromatin study demonstrating the non-linearity of fluorometry useful for calculating genomic base composition. Cytometry 14: 618–626. doi: 10.1002/cyto.990140606

Hall TJ (2001) History and development of *buffelgrass* pasture lands in Queensland. Proceedings of *Buffelgarss* Workshop. Theodore Qld, February 21–23, 2000. Departement of Primary Industries, Brisbane, Australia, 2–12.

Hamon P, Siljiak-Yakovlev S, Srisuwan S, Robin O, Poncet V, Hamon S, De Kochko A (2009) Physical mapping of rDNA and heterochromatin in chromosomes of 16 *Coffea* species: A revised view of species differenciation. Chromosome Research 17: 291–304. doi: 10.1007/s10577-009-9033-2

Hanson RE, Islam-Faridi MN, Percival EA, Crane CF, Ji Y, McKnight TD, Stelly DM, Price HJ (1996) Distribution of 5S and 18S–28S rDNA loci in a tetraploid cotton (*Gossypium*

hirsutum L.) and its putative diploid ancestors. Chromosoma 105: 55–61. doi: 10.1007/BF02510039

Heslop-Harrison JS (2000) Comparative genome organization in plants: from sequence and markers to chromatin and chromosomes. Plant Cell 12: 617–635. doi: 10.1105/tpc.12.5.617

Heslop-Harrison JS, Schwarzacher T, Anamthawat-Jónsson K, Leitch AR, Shi M, Leitch IJ (1991) *In situ* hybridization with automated chromosome denaturation. Technique 3: 109–115.

Jauffret S, Lavorel S (2003) Are plant functional types relevant to describe degradation in arid, southern Tunisian steppes? Journal of Vegetation Science 14: 399–408. doi: 10.1111/j.1654-1103.2003.tb02165.x

Jellen EN, Phillips RL, Rines HW (1988) Molecular genetic characterization of oat ribosomal DNAs. In: Agronomy Abstracts. CA: American Society of Agronomy, Anaheim, 169 pp.

Jiang J, Gill BS (2006) Current status and the future of fluorescence *in situ* hybridization (FISH) in plant genome research. Genome 49: 1057–1068. doi: 10.1139/G06-076

Kharrat-Souissi A, Siljak-Yakovlev S, Brown S, Chaieb M (in press) Cytogeography of *Cenchrus ciliaris* (Poaceae) in Tunisia. Folia Geobotanica.

Kharrat-Souissi A, Baumel A, Torre F, Juin M, Siljak-Yakovlev S, Roig A, Chaieb M (2011) New insights into the polyploid complex *Cenchrus ciliaris* L. (Poaceae) show its capacity for gene flow and recombination processes despite its apomictic nature. Australian Journal of Botany 59: 543–553. doi: 10.1071/BT10312

Kim NS, Kuspira J, Armstrong K, Bhambhani R (1993) Genetic and cytogenetic analyses of the A genome of *Triticum monococcum*. VII. Localization of rDNAs and characterization of the 5S rRNA genes. Genome 36:77–86. doi: 10.1139/g93-011

Le Houérou HN, Hoste CH (1977) Rangeland production and annual rainfall relationships in the Mediterranean basin and in the African Sahelo-Sudanian zone. Journal of Range Management 30: 181–189. doi: 10.2307/3897463

Le Houérou HN, Ionesco T (1973) L'appétabilité des espèces végétales de la Tunisie Steppique. Division Production et Protection des Plantes, FAO, Rome, 68 pp.

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromere position in chromosomes. Hereditas 52: 201–220. doi: 10.1111/j.1601-5223.1964.tb01953.x

Mortreau E, Siljak-Yakovlev S, Cerbah M, Brown SC, Bertrand H, Lambert C (2010) Cytogenetic characterization of *Hydrangea involucrata* Sieb. and *H. aspera* D. Don complex (Hydrangeaceae): genetic, evolutional, and taxonomic implications. Tree Genetics and Genomes 6: 137–148. doi: 10.1007/s11295-009-0235-8

Mseddi K, Mnif L, Chaieb M, Neffati M, Roux M (2004) Aboveground phytomass productivity and morphologic variability of Tunisian accessions of *Cenchrus ciliaris*. African Journal of Range and Forage Science 19: 13–20. doi: 10.2989/10220110409485833

Mukai Y, Endo TR, Gill BS (1991) Physical mapping of the 18S.26S rRNA multigene family in common wheat: identification of a new locus. Chromosoma 100: 71–78. doi: 10.1007/BF00418239

Muratović E, Bogunic F, Soljan D, Siljak-Yakovlev S (2005) Does *Lilium bosniacum* merit species rank? A classical and molecular cytogenetic approaches. Plant Systematic Evolution 252: 97–109. doi: 10.1007/s00606-004-0282-4

Muratović E, Robin O, Bogunic F, Soljan D, Siljak-Yakovlev S (2010) Speciation of European lilies from *Liriotypus* section based on karyotype evolution. Taxon 59: 165-175. http://www.ingentaconnect.com/content/iapt/tax/2010/00000059/00000001/art00016

Ozias-Akins P, Akiyama Y, Hanna WW (2003) Molecular characterization of the genomic region linked with apomixis in *Pennisetum*/*Cenchrus*. Functional and Integrative Genomics 3: 94–104. doi: 10.1007/s10142-003-0084-8

Pellicer J, Garnatje T, Molero J, Pustahija F, Siljak-Yakovlev S, Vallès J (2010) Origin and evolution of the South American endemic Artemisia species (*Asteraceae*): evidence from molecular phylogeny, ribosomal DNA and genome size data. Australian Journal of Botany 58: 605–616. doi: 10.1071/BT10047

Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58: 307–324. doi: 10.1007/BF00292840

Sherwood RT, Young BA, Bashaw EC (1980) Facultative apomixis in buffelgrass. Crop Science 20: 375–379. doi: 10.2135/cropsci1980.0011183X002000030022x

Siljak-Yakovlev S, Cerbah M, Coulaud J, Stoian V, Brown SC, Jelenic S, Papes D (2002) Nuclear DNA content, base composition, heterochromatin and rDNA in *Picea omorika* and *Picea abies*. Theoretical and Applied Genetics 104: 505–512. doi: 10.1007/s001220100755

Siljak-Yakovlev S, Paccenini S, Muratović E, Zoldos V, Robin O, Valles J (2003) Chromosomal differentiation and genome size in three European mountain *Lilium* species. Plant Systematic and Evolution 263: 165–173. doi: 10.1007/s00606-002-0240-y

Srisuwan S, Sihachakr D, Siljak-Yakovlev S (2006) The origin and evolution of sweet potato (*Ipomoea batatas* Lam.) and its wild relatives through the cytogenetic approaches. Plant Science 171: 424–433. doi: 10.1016/j.plantsci.2006.05.007

Stebbins GL (1971) Chromosomal evolution in higher plants. Addison-Wesley, Reading, Massachusetts, USA, 216 pp.

Stieber MT, Wipff JK (2003) *Cenchrus* L. In: Barkworth ME et al. (Eds) Flora of North America north of Mexico. Oxford University Press, New York, Oxford, 529–535.

Visser NC, Spies JJ, Venter HJT (2000) Apomictic embryo sac development in *Cenchrus ciliaris* (Panicoideae). Bothalia 30: 103–110.

Watson L, Dallwitz MJ (1992) The Grass Genera of the World. CAB International, Wallingford, 1038 pp.

Weiss-Schneeweiss H, Stuessy TF, Siljak-Yakovlev S, Baeza CM, Parker J (2003) Karyotype evolution in South American species of *Hypochaeris* (Asteraceae, Lactuceae). Plant Systematic and Evolution 241:171–184. doi: 10.1007/s00606-003-0026-x

Wendel JF (2000) Genome evolution in polyploids. Plant Molecular and Biology 42: 225–249. doi: 10.1023/A:1006392424384

CompCytogen 6(3): 287–300 (2012)
doi: 10.3897/CompCytogen.v6i3.3543
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Identifying parental chromosomes and genomic rearrangements in animal hybrid complexes of species with small genome size using Genomic *In Situ* Hybridization (GISH)

Massimiliano Rampin[1], Ke Bi[2], James P. Bogart[3], Maria João Collares-Pereira[1]

1 *Centro de Biologia Ambiental - Faculdade de Ciências - Universidade de Lisboa, Campo Grande - 1749-016 Lisboa, Portugal* **2** *Department of Integrative Biology, University of Guelph, Guelph, Ontario (Canada)* **3** *Museum of Vertebrate Zoology, University of California, Berkeley, California (USA)*

Corresponding author: *Massimiliano Rampin* (maxrampin@gmail.com)

Academic editor: *V. Lukhtanov* | Received 17 June 2012 | Accepted 9 August 2012 | Published 14 September 2012

Citation: Rampin M, Bi K, Bogart JP, Collares-Pereira MJ (2012) Identifying parental chromosomes and genomic rearrangements in animal hybrid complexes of species with small genome size using Genomic *In Situ* Hybridization (GISH). Comparative Cytogenetics 6(3): 287–300. doi: 10.3897/CompCytogen.v6i3.3543

Abstract

Genomic *In Situ* Hybridization (GISH) is a powerful tool to identify and to quantify genomic constituents in allopolyploids, and is mainly based on hybridization of highly and moderate repetitive sequences. In animals, as opposed to plants, GISH has not been widely used in part because there are technical problems in obtaining informative results. Using the allopolyploid *Squalius alburnoides* Steindachner, 1866 fish complex as a model system, we succeeded in overcoming methodological constraints when dealing with parental species with a small genome size. This hybridogenetic complex has biotypes with different genome compositions and ploidy levels, but parental chromosomes are small, morphologically very similar and therefore cannot be distinguished by conventional cytogenetic approaches. Specimens have a small genome (C-value1.2 pg) with a low level of highly and moderate repetitive sequences, mainly located at pericentromeric chromosome regions. Since it is well known that probe annealing depends on probe concentration and hybridization time to obtain uniform hybridization signals along the chromosome arms, we progressively increased the amount of labeled probes from 100ng up to 1µg and the incubation time from overnight up to 5 days. We also made other smaller improvements. Results showed a clear enhancement of signals with respect to previous data, allowing an accurate and reproducible assignment of the parental genomes in both diploid and triploid fish. It was thus evidenced that high probes' concentrations and long incubation time are the key to obtain, without extra image editing, uniform and reliable hybridization signals in metaphase chromosomes of animal hybrids from species with small genome size.

Keywords
Allopolyploids, GISH, Hybrids, C-value, fishes, *Squalius alburnoides* complex

Introduction

Genomic *In Situ* Hybridization (GISH) was developed by Schwarzacher et al. (1989) to identify parental chromosomes in *Hordeum chilense* Roemer et Schultes x *Secale africanum* Stapf hybrid plants where classical karyotyping and/or chromosome banding were unable to detect genomic (chromosomal) differences. This technique uses labeled total genomic DNA as probes to recognize a genome in chromosome preparations of hybrid individuals. It provides a straight and simple visual identification of parental chromosomes and genome organization (e.g. chromosomal rearrangements) in interspecific/intergeneric hybrids and allopolyploid species, as well as introgression, addition and substitution lines (see e.g. Jiang and Gill 1994, Jacobsen et al. 1995, Fujiwara et al. 1997, Sanchez-Moran et al. 1999, Bi and Bogart 2006).

GISH uses labeled total genomic DNA (gDNA) as the probe in *in situ* hybridization experiments together with sheared unlabeled whole genomic DNA, usually from the other parental species (but see Markova and Vyskot 2009) as blocking DNA. The blocking DNA serves as a DNA competitor to avoid the staining of both genomes by the probe DNA if the parents are closely related. GISH works primarily on hybridization of highly and moderate repetitive DNA sequences (Markova and Vyskot 2009 and included references). The required amount of blocking DNA is known to depend on the phylogenetic distance between the parental species and it can be reduced for distantly related parental species. Based on *Nicotiana* Linnaeus allopolyploids, Lim et al. (2007) established a parental species divergence from around 1MY to 5MY for obtaining reliable results by GISH. Thus it is possible to distinguish parental chromosomes in interspecific hybrids using GISH if the parental genomes are divergent and the hybridization is relatively recent (Markova and Vyskot 2009).

Hybridization and polyploidy are known to be common phenomena in plants. Recent genomic studies have revealed a higher occurrence of these events in animals than previously suspected (Gromicho and Collares-Pereira 2007, Mallet 2007, Mable et al. 2011). GISH has been a powerful tool for analyzing plant hybrids and polyploids (reviewed in Abbasi et al. 2010). However in vertebrates it has only been successfully used in unisexual salamanders (Bi and Bogart 2006, Bi et al. 2007a,b, 2008, 2009, 2010), in hybridogenetic frogs (Zalesna et al. 2011) and in a salmonid hybrid study (Fujiwara et al. 1997). There are two other GISH applications in fishes, but one only shows centromeric hybridization signals (Zhu and Gui 2007) and the other did not include any illustration of GISH results (Valente et al. 2009). The lack of more applications in animals may be explained by the difficulty in obtaining accurate and reproducible results due to technical problems related to genome size and distribution of genome-specific repetitive sequences, as suggested by Ali et al. (2004) for plants. The high quality GISH studies in vertebrates were performed in species complexes with large genomes (data from Greg-

ory 2011): *Ambystoma* Tschudi, 1838 (C-values ranging from 21.85pg-80.70pg) and *Pelophylax esculentus* Linnaeus, 1758 (5.60pg-11.53pg). Also in the *Oncorhynchus masou* Brevoort, 1856 x *Oncorhynchus mykiss* Walbaum, 1792 hybrid form, parental species have C-values ranging from 2.07pg-3.29pg and 1.87pg-2.92pg respectively, what may be considered relatively high in comparative terms for teleosts (reported C-values range from 0.35pg up to 4.90pg, mean value = 1.16pg – in Gregory 2011).

We aimed to improve GISH methodology using the *Squalius alburnoides* Iberian complex, as a model system. This fish complex originated from interspecific hybridization between *S. pyrenaicus* Günther, 1868 (P genome) as the maternal ancestor and a missing *Anaecypris*-like species (A genome) paternal ancestor. The complex is composed of different biotypes and ploidies (2n=50, 3n=75 and 4n=100), which are produced by non-sexual and sexual modes of reproduction. In southern Portugal, specimens carrying PA, PAA, PPA and PPAA genomes are found, as well as nuclear non-hybrid males AA which are reconstituted within the complex (reviewed in Gromicho and Collares-Pereira 2007, Collares-Pereira and Coelho 2010). Diploid specimens of *S. alburnoides* have a relatively small genome (C-value=1.2 pg; Próspero and Collares-Pereira 2000) and a low amount of highly and moderate repetitive sequences, mainly located at pericentromeric chromosome regions. Herein we describe an optimized protocol for obtaining reliable and informative results in hybrid fishes even if parental species have morphologically similar chromosome sets, relatively small chromosomes and C-values, which might be applicable to other animal hybrid genomes.

Materials and methods

Fish samples, ploidy and genome composition screenings

Chromosome preparations were obtained from cellular suspensions preserved in fixative (3:1 methanol:acetic acid) at -20°C for about 6 years from five diploid (PA genome, 2n=50) and one triploid (PAA genome, 3n=75) *S. alburnoides* specimens. The fish were selected from the offspring of artificial crosses obtained using specimens captured at Guadiana river basin in 2001–2003. Several specimens with an unknown genomic composition were also collected by electrofishing in October 2010 at Almargem drainage. Both populations are in Southern Portugal. The best two cellular suspensions from this pool (of two distinct triploid females) were also used for GISH experiments. Probe and blocking DNA were obtained from nuclear non-hybrid specimens of *S. alburnoides* (AA genome) and allopatric specimens of *S. pyrenaicus* (PP genome), respectively.

Ploidy of the fish was determined by analysis of erythrocyte DNA content using a Coulter Epics XL cytometer, following the method described in Collares-Pereira and Moreira da Costa (1999). Genome composition of the old suspensions was assessed following Crespo-López et al. (2006), and biotypes of the new sampled specimens were identified using the method of Sousa-Santos et al. (2005).

Chromosomes preparation

Metaphase chromosomes were obtained from fibroblast fin cultures according to the method of Rodrigues and Collares Pereira (1996) with small modifications. After checking the quantity and quality of metaphases, the best suspensions were used for GISH experiments. In order to improve spreading of the metaphase plates, the slides were placed on a wet, cold (+4°C) sponge and one or two drops of the suspension were released onto high quality superfrost precleaned glass slides (Cole – Parmer, Vernon Hills Illinois). This treatment increases the surface tension allowing better separation of the chromosomes (open plates). The quality of the chromosome spreads was evaluated by phase-contrast microscopy for subsequent experiments. Selection criteria were: high number of metaphase plates, well separated chromosomes with few or no overlapping chromosome arms and little cytoplasm surrounding the plates. The slides were aged 2 to 5 days at room temperature (RT) or overnight at 70°C.

Labeled probe preparation

Total genomic DNA (gDNA) from *S. pyrenaicus* and *S. alburnoides* nuclear non-hybrid males were extracted from muscle and fins using Phenol:Chloroform:Isoamyl alcohol (PCI) method (Sambrook et al. 1989). gDNA quality was assessed by agarose gel electrophoresis. Completely degraded DNA samples are not optimal to obtain a good labeled probe, even though the manufacturer's protocol (Roche) suggests using fragmented DNA. The amount of gDNA was evaluated at first using NanoDrop 1000 (Fisher Scientific) by diluting the samples in double-distilled H_2O 1:100, and subsequently using both NanoDrop and a QBit Fluorometer (Invitrogen). The probe was labeled with Dig-11-dUTP according to the manufacturer's protocol (Roche cat N° 11745816910) with small modifications. The amount of starting DNA was increased to 1.3–1.5µg and the incubation time was extended to 135 - 150 min in order to obtain a greater amount of labeled probe. Quality and quantity were assessed. After precipitation and air-drying, the probe was re-suspended in 40µl of GISH mix, a solution composed of 50% Dextran Sulphate (Promega), 10% SDS, 2× SSC, 500 ng/µl sheared salmon sperm DNA, 50x Denhardt's and double-distilled H_2O (final concentration of probe DNA 25ng/µl) during the first experiments (Set I, see below). In a second set of experiments (Set II), the labeled probe was re-suspended in Hybridization Mix composed of 50% Ultra Pure Formamide (Sigma F9037), 2× SSC, 10% Dextran Sulphate, and Milli-Q water; pH adjusted to 7–7.5 with 1N HCl. The initial probe DNA concentration was 20 ng/µl, but was later increased up to 200 ng/µl.

Blocking DNA preparation

Genomic DNA for unlabeled blocking DNA was extracted from muscle and fins of non-hybrid samples (both AA and PP) using PCI method as described above. Several

individuals were used in order to obtain a large amount of gDNA. After air-drying, the DNA was re-suspended in double-distilled H_2O and the gDNA quality assessed by agarose gel electrophoresis. In this case it is not important if it is partially degraded. The DNA was then precipitated, air-dried and re-suspended in double-distilled H_2O, vortexed and accumulated in a single tube. A hole was punched in the tube lid, and the suspension was autoclaved for 40 min. After autoclaving, an agarose gel electrophoresis test was performed to evaluate the rate of DNA shearing. The optimal size fragments for an efficient blocking DNA ranges from 100bp to 1000bp. Sheared DNA was then concentrated using a speed vacuum centrifuge (to about 5 μg/μl), and evaluated as described above.

Genomic *In Situ* Hybridization (GISH)

Two sets of experiments (I and II) were performed.

Set I experiments (standard protocol)

The first experiments were performed according to the protocol used by Bi and Bogart (2006). Chromosomes were derived from old cell suspensions from diploid (PA) and triploid (PAA) specimens that were stored in fixative at -20°C. Slide quality was evaluated as previously described and hybridization areas on the slide were identified using a diamond pen. The slides were aged for five days at RT. After aging, chromosomes were denatured for 2 min in Formamide 70%, 2× SSC at 72°C-74°C. The slides were then immediately dehydrated in a series of ice cold alcohols: 70% EtOH (7 min), 90% EtOH (7 min), 100% EtOH (10 min) and then air-dried.

Probe mix preparation

For each slide, the labeled hybridization probe mix was: 10μl Ultra-Pure Formamide (Sigma F9037), 5μl GISH mix, and 5μl Dig labeled probe (about 100ng). A range from 1:0 to 1:30 probe:blocking DNA (P/B ratio) was added to these solutions to optimize genomic differentiation. The solutions were stored at 4°C prior to denaturation.

Probe denaturation and hybridization

During slide air-drying, the probe mixes were denatured for 7 min at 83°C in a PCR thermal cycler then immediately placed on ice for at least 10 min. 20 μl of probe mix was dropped onto each slide, a 22x22 mm coverslip was applied, sealed with parafilm, and incubated overnight at 37°C in a moist chamber.

Post-hybridization washes

Parafilm was removed and slides were immersed in 50% Formamide at 42°C for 10 min followed by three washes in 1× SSC for 5–7 min each at 42°C with gentle hand shaking. The washing steps were followed by rinsing in 2× SSC. To block unspecific

binding sites in order to reduce fluorescence in the background, 70µl of Blocking Solution (BSA 5%, 20× SSC, double-distilled H_2O and 0.1% Tween20) was applied to each slide, cover slips were then re-applied, and slides were incubated for 20 min at 37°C in a moist chamber.

Detection solution

Detection solution was made by diluting fluorescent antibody Anti-Digoxigenin FITC 1:100 in double-distilled H_2O. 30µl of this solution was applied onto each slide, covered with a 22x22 mm coverslip and incubated for 80 min at 37°C in a moist chamber. The coverslip was then removed and the slide washed 4x 5 min in pre-warmed Washing Buffer (4× SSC, 0.1% Tween20) at 42°C. Then slides were rinsed in 2× SSC and counterstained using 1.5µg/ml Propidium Iodide (PI) or 1.5µg/ml DAPI (4',6-diamidino-2-phenylindole) in antifade solution (Vectashield H-1300 and H-1200 respectively). Finally the slides were covered by a 24x32 mm coverslip and sealed with nail polish.

Microscope analysis

Slides were analyzed using a Leica DMRB fluorescence microscope. Fluorescent images from FITC, PI and DAPI were captured by a CCD camera (QImagine, Vancouver, Canada) and merged using Openlab 3.5 Software. All the images were analyzed and slightly manipulated with Adobe® Photoshop Elements 6 and Adobe® Photoshop CS4.

Set II experiments (GISH protocol optimization and validation)

Several parameters were modified in order to improve *in situ* hybridization quality, which is important for an accurate interpretation of GISH results. All the modifications were introduced step by step, and other experimental conditions remained constant. The old cell suspensions that provided chromosomes were initially used in parallel with the two new cell suspensions, and every modification was introduced as a fixed parameter in the following trials.

Water type

The first optimization experiments were performed using the same chromosome suspensions in the same conditions changing only the water type from di-deionized to Milli-Q Millipore® (18.2 MΩ·cm) in all the solutions.

Pepsin pretreatment (optional)

Pepsin pretreatment was performed before the denaturation but only on the slides in which metaphase spreads were surrounded by abundant cytoplasm. Slides were placed 10–12 min in pre-warmed (37°C) 0.01N HCl containing 0.002% pepsin (Sigma P7012). Pepsin activity was stopped by immersion in 1× PBS pH 7.4 for 5 min with gentle shaking, once in 2× SSC 5 min then dehydrated in a series of alcohol (70%, 90%, 100%) at RT.

Denaturation temperature

In order to preserve chromosomes morphology, denaturation temperature was decreased to 65°C and denaturation time extended from 2 minutes up to 3 minutes.

Post-hybridization washes

Post-hybridization washes were modified using 2 times 2× SSC, once 2× SSC+0.1% Tween20 and 1× PBST/0.5% w/v powder skimmed milk (as blocking solution). All these washes were performed for 5 min with gentle shaking at 42°C. In the following experiments these washes were performed at RT in order to reduce the stringency.

Probe re-suspension mixture

The labeled probe was re-suspended in Hybridization Mix composed of 50% Ultra-Pure Formamide (Sigma F9037), 2× SSC, 10% Dextran Sulphate (Promega), and Milli-Q water; pH adjusted to 7–7.5. The final concentration was 40–50ng/μl, and later increased up to 200 ng/μl.

Incubation times

The incubation time was progressively extended from overnight up to 5 days (see methods in Ali et al. 2004), though the best results were obtained with an incubation time of around 72 h.

Quantity of Probe

Initially, 100 ng of probe in a 22x22 mm hybridization area were used. Then the amount of the probe was increased to 200 ng, 500 ng, 1μg and a little bit more. Having suspensions containing many well-spread metaphases, the hybridization area was reduced to 10x10 mm, 12x12 mm or a 13 mm diameter. In these cases we applied from 200 to 350 ng of labeled probe on each slide.

P/B ratio evaluation

Probe/blocking DNA ratio was altered but used the same amount of labeled probe (1μg based on the previous tests) and the P/B ratio ranged from 1:20 to 1:40.

Pre-annealing test

In order to reduce the intensity of hybridization signals from shared highly repetitive sequences (e.g. rDNA, centromeric heterochromatin), a pre-annealing step of 30 min incubation at 37°C was introduced after probe denaturation (in a thermal cycler).

Microscope analysis

Slides were analyzed using an Olympus BX 60 fluorescence microscope. Fluorescent images from FITC, PI and DAPI staining were captured with an Olympus DP50 CCD camera. The images were analyzed and slightly manipulated with Adobe® Photoshop CS4.

Results

The first GISH experiments (Set I) on PA and PAA metaphase plates from old chromosome suspensions using the standard protocol of Bi and Bogart (2006), were made by applying 100 ng of Dig labeled AA whole genomic probe in presence of an excess of PP blocking DNA (P/B ratio from 1:0 to 1:30). Probe fluorescence was revealed by anti-digoxigenin FITC-conjugated antibody. The results showed non uniform, faint and spotted hybridization signals, mainly at pericentromeric chromosome regions and rDNA clusters as well as in some heterochromatic regions (Fig. 1a–d). For PA, only

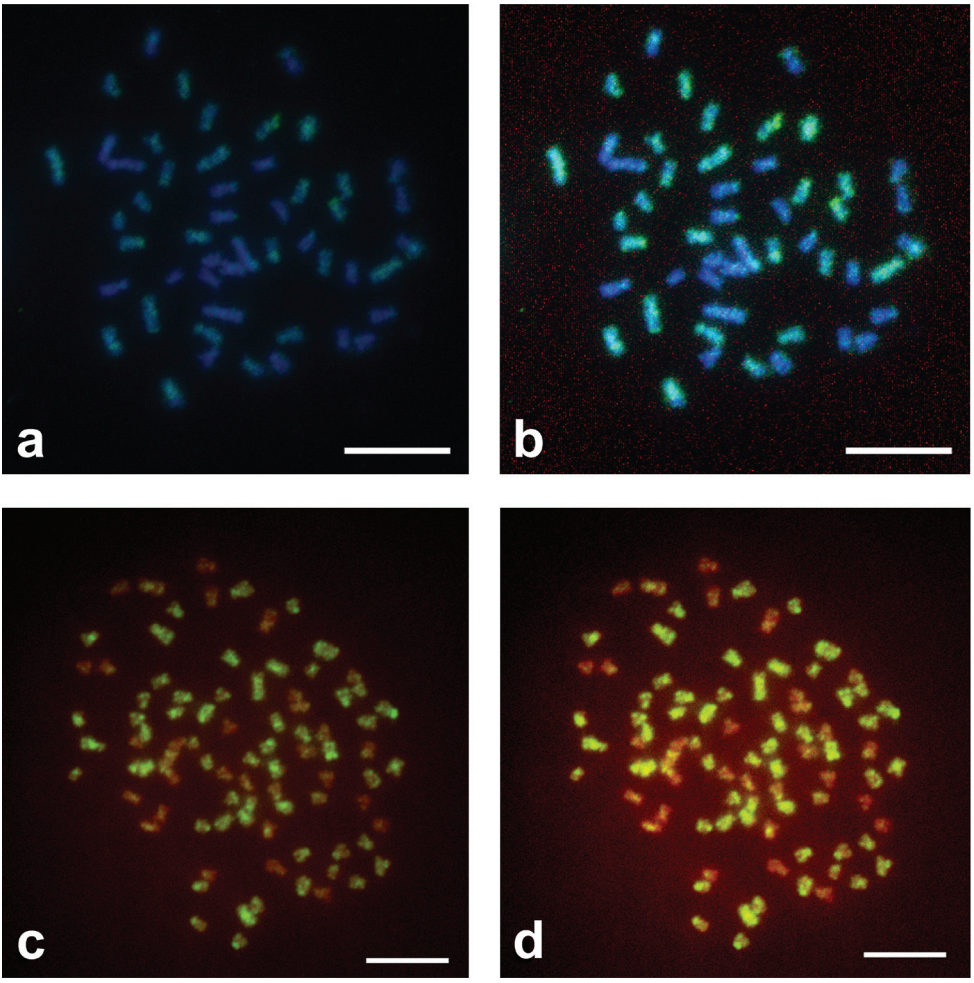


Figure 1. Standard use of GISH method in metaphase plates of specimens of *S. alburnoides* complex. **a** GISH with AA Dig labeled probe in PA metaphase plate revealed by anti-Dig FITC antibody counterstained with DAPI - picture without photo editing **b** the same picture with photo editing **c** GISH with AA Dig labeled probe in PAA metaphase plate revealed by anti-Dig FITC antibody counterstained with PI, picture without photo editing **d** the same picture with photo editing. Scale Bars = 10μm.

5 or 6 chromosomes were uniformly stained and the fluorescence was faint (Fig. 1a and 1c). It was difficult to correctly identify all the A genome chromosomes in the PA diploid hybrid on the basis of centromeric signals. Also, in the PAA plates (Fig. 1c and 1d), hybridization signals were faint and it was more or less impossible to correctly identify or to distinguish A from P chromosomes. It was also not possible to evaluate P/B ratio differences because the results obtained when changing blocking DNA amount were more or less equivalent (not shown).

The results from Set II experiments improved significantly by changing water type, post-hybridization washes, probe re-suspension mixture, pepsin pretreatment and fresh suspensions. MilliQ water contains fewer minerals affecting the hybridization of the probe. It stabilizes better hydrogen bonds between the probe and chromosomal DNA. Post-hybridization washes' modification reduced the stringency for the same reason. Pepsin pretreatment reduced fluorescent background, though no remarkable improvement was observed on hybridization quality. Reliable results were obtained when we used fresh chromosome suspensions, 65 h to 72 h incubation times and 1μg or more labeled probe per slide as shown in Figure 2a-2d. Uniform hybridization allowed to correctly identify parental chromosomes. Even when stronger signals occurred in rDNA clusters and at pericentromeric regions it was still possible to identify a few interchromosomal exchanges (Fig. 2d'). The optimal P/B ratio was 1:25. For suspensions containing a high number of metaphase plates, hybridization area reduction gave the same positive results (best size 12x12 mm or 13 mm diameter). This reduced the required amount of expensive labeled probe. In this set of experiments, little or no image editing was required to improve image quality.

Discussion

When comparing the two sets of experiments and the improvements introduced to the protocol provided by Bi and Bogart (2006), it is evident that the metaphase chromosomes of the PA and PAA hybrids after GISH did not give reliable and informative results (Fig. 1a–d). Only the pericentromeric heterochromatin and some chromosomes of one of the two parental genomes could be identified but without absolute certainty. In most of the diploid and triploid hybrid plates a strong hybridization signal was observed at pericentromeric regions. Hybridization sites likely corresponded to heterochromatic areas showed by C-banding, which usually contain repetitive sequences, but it was not uniform and was often faint even in some centromeres. It was therefore impossible to clearly distinguish the two parental chromosomes sets. This problem was likely the result of a low concentration of the probe but also of the incorrect amount of blocking DNA.

Even though metaphase chromosomes used in Set I experiments appeared to be of very good quality and very clean when assessed by phase-contrast microscopy, the lack of genome specific fluorescence may be explained by the age of the cell suspensions as has been demonstrated both in plants and animals. Physical and chemical molecular modifications likely affect chromosome quality, and the use of fresh suspensions is always preferable (e.g. Henegariu et al. 2001).

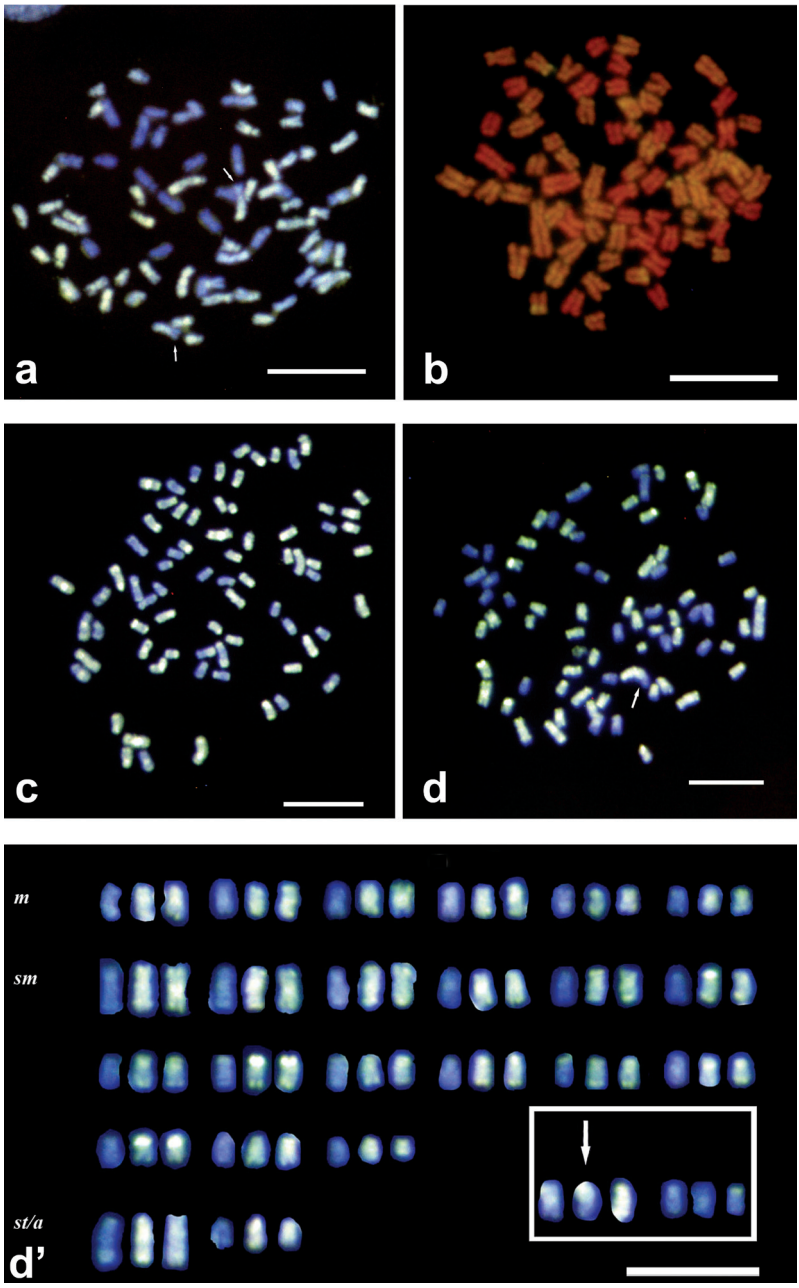


Figure 2. Improved use of GISH method and putative karyotype in specimens of *S. alburnoides* complex **a** GISH with AA Dig labeled probe in PAA metaphase plate revealed by anti-Dig FITC antibody counterstained with DAPI. Picture without photo editing. First improvement step **b-d** GISH with AA Dig labeled probe in PAA metaphase plates revealed by anti-Dig FITC antibody counterstained with DAPI (**c** and **d**) and PI (**b**). Pictures with little photo editing. Last improvement step **d'** Putative PAA karyotype. Arrows indicate chromosomes putatively involved in exchanges, and box in karyotype some unresolved chromosomes. Scale Bars = 10μm.

Our experiments revealed other aspects that likely contributed to improve the quality of hybridization, such as those related to renaturation kinetics and the concentration of the probe. Hybridization kinetics that drives the formation of hetero- and/or homoduplex complexes depends on several factors: genome size, sequences copy number, DNA fragment size, base composition, concentration and time (Wetmur and Davidson 1968, Werman et al. 1996 and references therein). Salamanders of the genus *Ambystoma* have quite large genomes (22pg<GS>81pg, Gregory 2011). Coding sequences likely represent a very small fraction of the genome, which is mainly constituted by non-coding DNA including highly, moderate and interspersed repetitive sequences. Thus, in organisms with large genomes, reliable GISH results can be obtained with a short incubation time (overnight) and only a small amount of labeled probe is necessary (100ng or less). On the other hand, specimens from the *S. alburnoides* complex have a much lower C-value (about 1.2pg; Próspero and Collares-Pereira 2000). Consequently, considering renaturation kinetics theory, hybridization likely requires much higher probe concentrations (C_0) and much longer incubation times (t).

Set II experiments increased probe concentration by reducing the hybridization area. A 22x22 mm area corresponds to 484 mm^2. If 1µg of probe is used in this area it means that a Weight Specific Surface Area is about 2ng/mm^2. Thus, operating on a reduced area, e.g. 100 mm^2, it will be sufficient to drop only 200ng of the probe instead of 1µg. By adopting this system of hybridization area reduction it is possible to increase the concentration of the probe without using a higher amount of probe. The best results obtained with this procedure (Fig. 2b-2d) used 300ng of labeled probe in 4µl of Hybridization Mix in areas of 12x12 mm or 13 mm diameter (P/B ratio 1:25).

Improved protocol provided uniform and clean hybridization without any, or very little, requirement for image editing using more than 1µg of labeled probe and about 72 h of incubation in moist chamber at 37°C.

A few of the chromosomes contained mixed fluorescence along the arms (Fig. 2c–d), suggesting the occurrence of intergenomic exchanges (Rampin et al. 2010). However, though a tentative karyotype was built for distinct triploid (PAA) females, since parental chromosome sets are grossly very similar and constituted by a high number of relatively small chromosomes, we can not confirm the patterns of exchanges and identify the individual chromosomes that are involved (Fig. 2d').

In vertebrates, intergenomic interactions have only been demonstrated by GISH in allopolyploid salamanders (Bi et al. 2007a, 2008, 2009, 2010). According to Zalesna et al. (2011) there were no such evidences in the hybridogenetic *Pelophylax esculentus* though only diploid hybrids were analysed. The results we have obtained confirm the power of GISH to define genomic composition in hybrids and to visualize interchromosomal exchanges (if present) even when dealing with hybrids of parental species having small chromosomes and C-values. Thus with the described improvements, GISH methodology will likely have a much wider application and be very helpful for cytogeneticists, when coupled with other molecular approaches, to unravel genome restructuring processes also in animal hybrid complexes.

Acknowledgements

We thank DGRF for fishing permits and all the CBA Evolutionary Genetics Group for help both in the field and in the lab work processing. This research was mainly funded by the FCT Pluriannual Program (UI&D 329/94). MR was supported by the FCT Post-Doctoral grant SFRH/BPD/48428/2008.

References

Abbasi FM, Tarik-Khan M, Perveen F, Masood R, Uzma.Khan I, Tabassum S, Shah M (2010) Historical perspective of *in situ* hybridization for the analysis of genomic constitution of plants. African Journal of Biotechnology 9: 9142–9147

Ali BM, Lysak MA, Schubert I (2004) Genomic *in situ* hybridization in plants with small genomes is feasible and elucidates the chromosomal parentage in interspecific *Arabidopsis* hybrids. Genome 47: 954–960. doi: 10.1139/g04-041

Bi K, Bogart JP (2006) Identification of intergenomic recombinations in unisexual salamanders of the genus *Ambystoma* by genomic *in situ* hybridization (GISH). Cytogenetic and Genome Research 112: 307–312. doi: 10.1159/000089885

Bi K, Bogart JP, Fu J (2007a) Intergenomic translocations in unisexual salamanders of the genus *Ambystoma* (Amphibia, Caudata). Cytogenetic and Genome Research 116: 289–297. doi: 10.1159/000100413

Bi K, Bogart JP, Fu J (2007b) Two rare aneutriploids in the unisexual *Ambystoma* (Amphibia, Caudata) identified by GISH indicating two different types of meiotic errors. Cytogenetic and Genome Research 119: 127–130. doi: 10.1159/000109628

Bi K, Bogart JP, Fu J (2008) Genealogical relationships of southern Ontario polyploid unisexual salamanders (genus *Ambystoma*) inferred from intergenomic exchanges and major rDNA cytotypes. Chromosome Research 16: 275–289. doi: 10.1007/s10577-007-1192-4

Bi K, Bogart JP, Fu J (2009) An examination of intergenomic exchanges in *A. laterale*-dependent unisexual salamanders in the genus *Ambystoma*. Cytogenetic and Genome Research 124: 44–50. doi: 10.1159/000200087

Bi K, Bogart JP (2010) Probing the meiotic mechanism of intergenomic exchanges by genomic in situ hybridization on lampbrush chromosomes of unisexual *Ambystoma* (Amphibia: Caudata). Chromosome Research 18: 371–82. doi: 10.1007/s10577-010-9121-3

Collares-Pereira MJ, Moreira da Costa L (1999) Intraspecific and interspecific genome size variation in Iberian Cyprinidae and the problem of diploidy and polyploidy, with review of genome sizes within the family. Folia Zoologica 48: 61–76.

Collares-Pereira MJ, Coelho MM (2010) Reconfirming the hybrid origin and generic status of the Iberian cyprinid complex *Squalius alburnoides*. Journal of Fish Biology 76(3): 707–715. doi: 10.1111/j.1095-8649.2009.02460.x

Crespo-López E, Duarte T, Dowling T, Coelho MM (2006) Modes of reproduction of the hybridogenetic fish *Squalius alburnoides* in Tejo and Guadiana rivers: an approach with microsatellites. Zoology 109: 277–286. doi: 10.1016/j.zool.2006.03.008

Fujiwara A, Abe S, Yamaha E, Yamazaki F, Yoshida MC (1997) Uniparental chromosome elimination in the early embryogenesis of the inviable salmonid hybrids between masu salmon female and rainbow trout male. Chromosoma 106: 44–52. doi: 10.1007/s004120050223

Gregory TR (2011) Animal Genome Size Database. [http://www.genomesize.com]

Gromicho M, Collares-Pereira MJ (2007) The evolutionary role of hybridization and polyploidy in an Iberian cyprinid fish – a cytogenetic review. In: Pisano E, Ozouf-Costaz C, Foresti F, Kapoor BG (Eds) Fish Cytogenetics. Oxford & IBH Publishing, 41–67.

Henegariu O, Heerema NA, Wright LL, Bray-Ward P, Ward DC, Vance GH (2001) Improvements in cytogenetic slide preparation: controlled chromosome spreading, chemical aging and gradual denaturing. Cytometry 43: 101–109. doi: 10.1002/1097-0320(20010201)43:2<101::AID-CYTO1024>3.0.CO;2-8

Jacobsen E, De Jong JH, Kamstra SA, Van den Berg, PM, Ramanna MS (1995) Genomic *in situ* hybridization (GISH) and RFLP analysis for the identification of alien chromosomes in the backcross progeny of potato (+) tomato fusion hybrids. Heredity 74: 250–257. doi: 10.1038/hdy.1995.38

Jiang J, Gill BS (1994) Different species-specific chromosome translocations in *Triticum timopheevii* and *T. turgidum* support the diphyletic origin of polyploid wheats. Chromosome Research 2: 59–64. doi: 10.1007/BF01539455

Lim KY, Kovarik A, Matyasek R, Chase MW, Clarkson J, Grandbastien MA, Leitch AR (2007) Sequence of events leading to near-complete genome turnover in allopolyploid *Nicotiana* within five millionyears. New Phytologist 175: 756–763. doi: 10.1111/j.1469-8137.2007.02121.x

Mable BK, Alexandrou MA, Taylor MI (2011) Genome duplication in amphibians and fish: an extended synthesis. Journal of Zoology 284: 151–182. doi: 10.1111/j.1469-7998.2011.00829.x

Mallet J (2007) Hybrid speciation. Nature 446: 279–283. doi: 10.1038/nature05706

Markova M, Vyskot B (2009) New horizons of genomic *in situ* hybridization. Cytogenetic and Genome Research 126: 368–375. doi: 10.1159/000275796

Próspero MI, Collares-Pereira MJ (2000) Nuclear DNA content variation in the diploid-polyploid *Leuciscus alburnoides* complex (Teleostei, Cyprinidae) assessed by flow cytometry. Folia Zoologica 49: 53–58

Rampin M, Bi K, Bogart J, Collares-Pereira MJ (2010) Identification of putative chromosomal exchanges in *S. alburnoides* non-sexual fish complex by genomic *in situ* hybridization (GISH): a confirmation of preliminary results. Chromosome Research 18: 752–753. [abstract]

Rodrigues EM, Collares-Pereira MJ (1996) NOR polymorphism in the Iberian species *Chondrostoma lusitanicum* (Pisces: Cyprinidae). Genetica 98: 59–63. doi: 10.1023/A:1003885922023

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A Laboratory Manual, 2nd Edition, Vol. 3, Cold Spring Harbor Laboratory Press, E3–E4.

Sanchez-Moran E, Benavente E, Orellana J (1999) Simultaneous identification of A, B, D and R genomes by genomic *in situ* hybridization in wheat-rye derivatives. Heredity 83: 249–252. doi: 10.1038/sj.hdy.6885570

Schwarzacher T, Leitch AR, Bennett MD, Heslop-Harrison JS (1989) *In situ* localization of parental genomes in a wide hybrid. Annals of Botany 64: 315–324.

Sousa-Santos C, Robalo JI, Collares-Pereira MJ, Almada VC (2005) Heterozygous indels as useful tools in the reconstruction of DNA sequences and in the assessment of ploidy level and genomic constitution of hybrid organisms. DNA Sequence 16: 462–467. doi: 10.1080/10425170500356065

Valente TG, Schneider HC, Gross CM, Feldberg E, Martins C (2009) Comparative cytogenetics of cichlid fishes through genomic *in situ* hybridization (GISH) with emphasis on *Oreochromis niloticus*. Chromosome Research 17: 791–799. doi: 10.1007/s10577-009-9067-5

Werman SD, Springer MS, Britten RJ (1996) Nucleic Acids I: DNA-DNA Hybridization. In: Hillis DM, Moritz C, Mable BK (Eds) Molecular Systematics. 2nd Edition. Sinauer Associates, Sunderland, MA, 168–203.

Wetmur JG, Davidson N (1968) Kinetics of renaturation of DNA. Journal of Molecular Biology 31: 349–70. doi: 10.1016/0022-2836(68)90414-2

Zalesna A, Choleva L, Ogielska M, Rábová M, Marec F, Ráb P (2011) Evidence for integrity of parental genomes in the diploid hybridogenetic water frog *Pelophylax esculentus* by Genomic *in situ* Hybridization. Cytogenetetic and Genome Research 134: 206–212. doi: 10.1159/000327716

Zhu H-P, Gui J-F (2007) Identification of genome organization in the unusual allotetraploid form of *Carassius auratus gibelio*. Aquaculture 265: 109–117. doi: 10.1016/j.aquaculture.2006.10.026

CompCytogen 6(3): 301–314 (2012)
doi: 10.3897/CompCytogen.v6i3.3701
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Synaptonemal complex analysis of interracial hybrids between the Moscow and Neroosa chromosomal races of the common shrew *Sorex araneus* showing regular formation of a complex meiotic configuration (ring-of-four)

Sergey N. Matveevsky[1], Svetlana V. Pavlova[2], Maret M. Acaeva[1,3], Oxana L. Kolomiets[1]

1 *N.I. Vavilov Institute of General Genetics, Russian Academy of Sciences, Gubkin str. 3, Moscow 119991, Russia* **2** *A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Leninskiy pr. 33, Moscow 119071, Russia* **3** *Chehen State University, A. Sheripov str. 32, Grozny 364051, Chechen Republic, Russia*

Corresponding author: *Sergey N. Matveevsky* (sergey8585@mail.ru)

Academic editor: *I. Bakloushinskaya* | Received 19 July 2012 | Accepted 24 August 2012 | Published 14 September 2012

Citation: Matveevsky SN, Pavlova SV, Acaeva MM, Kolomiets OL (2012) Synaptonemal complex analysis of interracial hybrids between the Moscow and Neroosa chromosomal races of the common shrew *Sorex araneus* showing regular formation of a complex meiotic configuration (ring-of-four). Comparative Cytogenetics 6(3): 301–314. doi: 10.3897/CompCytogen.v6i3.3701

Abstract

Immunocytochemical and electron microscopic analysis of synaptonemal complexes (SCs) was carried out for the first time in homozygotes and complex Robertsonian heterozygotes (hybrids) of the common shrew, *Sorex araneus* Linnaeus, 1758, from a newly discovered hybrid zone between the Moscow and the Neroosa chromosomal races. These races differ in four monobrachial homologous metacentrics, and closed SC tetravalent is expected to be formed in meiosis of a hybrid. Indeed, such a multivalent was found at meiotic prophase I in hybrids. Interactions between multivalent and both autosomes and/or the sex chromosomes were observed. For the first time we have used immunocytochemical techniques to analyse asynapsis in *S. araneus* and show that the multivalent pairs in an orderly fashion with complete synapsis. Despite some signs of spermatocytes arrested in the meiotic prophase I, hybrids had large number of active sperm. Thus, Moscow – Neroosa hybrid males that form a ring-of-four meiotic configuration are most likely not sterile. Our results support previous demonstrations that monobrachial homology of metacentrics of the common shrew does not lead to complete reproductive isolation between parapatric chromosomal races of the species.

Keywords
Synaptonemal complex, MSCI, γH2AX, centromeres, *Sorex araneus*

Introduction

The concept of chromosomal speciation implies occurrence of reproductive isolation as a result of chromosomal rearrangements (Vorontsov 1960, White 1978, 1982, King 1993). The most common type of chromosome rearrangements in mammalian evolution is represented by the Robertsonian translocations – fusion of two acrocentric chromosomes into a single submeta – or metacentric chromosome. It was first described in orthopterous insects (Robertson 1916). Species with a so-called "Robertsonian fan" represent unique models for studying chromosomal speciation. The term was introduced by R. Matthey for description of a wide-range chromosomal variation caused by multiple Robertsonian translocations (Matthey 1970). Among mammals, there are several species that demonstrate the Robertsonian fan: the Subsaharan pygmy mouse, *Mus (Nannomys) musculoides* Temminck, 1853 (Matthey 1970, Jotterand 1972), the house mouse, *Mus musculus domesticus* Schwarz et Schwarz, 1943 (Gropp et al. 1972, Gropp and Winking 1981), the Eastern mole vole, *Ellobius tancrei* Blasius, 1884 (Lyapunova et al. 1980), the Nigerian gerbil, *Gerbillus nigeriae* Thomas et Hinton, 1920 (Volobouev et al. 1988), the Daghestan pine vole, *Pitymys daghestanicus* Shidlovsky, 1919 (Tembotov et al. 1976), and the common shrew, *Sorex araneus* Linnaeus, 1758 (Searle and Wójcik 1998).

Due to its high level of karyotype variability, the common shrew *S. araneus* is subdivided into at least 72 parapatric chromosomal races, each characterised by a unique set of metacentric chromosomes formed by Robertsonian fusions and/or whole-arm reciprocal translocations (WARTs) (Hausser et al. 1994, White et al. 2010). Three metacentrics (*af*, *bc*, *tu*) and sex chromosomes (XX in females and XY_1Y_2 trivalent in males) are invariant in all chromosomal races, while another ten autosomal arms (*g-r*) may occur as acrocentrics and/or combined together as metacentrics (Searle et al. 1991). XY_1Y_2 sex trivalent formed by the X-autosome translocation (Sharman 1956) is specific for the species of the '*Sorex araneus*' group (Zima et al. 1998). The sex trivalent has original parts (*e* "true" arm of X chromosome and the Y_1) and autosomal parts (*d* translocated arm of X chromosome homologous to the Y_2) (Fredga 1970, Pack et al. 1993).

Hybrids between parapatric chromosomal races of the common shrew are often expected to be complex Robertsonian heterozygotes with monobrachial homology, which form chain (C) or ring (R) configurations of three or more elements at prophase I of meiosis. Such complex meiotic configurations are considered to be more susceptible to irregularity. As a consequence, complex heterozygotes are expected to be less fertile than homozygotes of pure chromosomal races. At present, interracial hybrids with different types of meiotic configurations from CIII and RIV up to CXI and RVI have been revealed from seventeen well-studied hybrid zones (Searle and Wójcik 1998, Bulatova et al. 2011, Polyakov et al. 2011, Orlov et al. 2012). Studies so far have shown that hybrids with long chain or ring configurations have more abnormalities

during meiosis than hybrids with shorter configurations; however even in these cases the complex meiotic configurations do not appear to be associated with complete sterility (Mercer et al. 1992, Narain and Fredga 1997, Jadwiszczak and Banaszek 2006, Pavlova et al. 2008). There is a need to document more fully the match between complexity of karyotype and degree of regularity of the meiotic configurations expected.

A new chromosomal hybrid zone between the Moscow race (***gm***, *hi*, *jl*, *kr*, ***no***, *pq*, *2n*a=18) and the Neroosa race (***go***, *hi*, *jl*, *kr*, ***mn***, *pq*, *2n*a=18) has been found recently in the centre of European Russia (Pavlova et al. 2012, *in press*). Karyotypes of the races differ in four metacentrics with monobrachial homology so that the complex heterozygotes should form a ring-of-four (RIV) configuration at meiosis I. On the basis of karyotype differences of the races one can suggest that fixation of just one WART, between metacentrics *gm* and *no* or between *go* and *mn*, could have separated these races in the past.

This paper presents a comparative synaptonemal complex (SC) analysis of prophase I of meiosis using electron microscopy and immunofluorescence in homozygotes and complex Robertsonian heterozygotes from this hybrid zone. A combination of both methods together for SC analysis is used for the first time in *Sorex araneus*.

Material and methods

Animals and karyotypes. A total of eight adult male common shrews were collected from the Moscow-Neroosa hybrid zone, located in the south-eastern part of the Moscow Region near Ozyory town (the left bank of the River Oka), in April 2012, at the beginning of the breeding season. Each specimen was processed according to the field procedure described in Bulatova et al. (2009). Mitotic chromosomes were prepared from bone marrow and spleen following Ford and Hamerton (1956) with modifications. A trypsin - Giemsa staining technique of Kràl & Radjabli (1974) was used for identification of chromosome arms according to the standard nomenclature for the *S. araneus* karyotype (Searle et al. 1991). Only three of eight karyotyped males were used for the meiotic analysis.

Synaptonemal Complex Analysis. Synaptonemal complex (SC) preparations were prepared and fixed using the technique of Navarro et al. (1981) with modifications (Kolomiets et al. 2010). Measurements of autosomal bivalents and their ranking in each cell were made in order to determining relative lengths (MicroMeasure 3.3, Colorado, USA).

Electron microscopy. Slides were stained with a 50% $AgNO_3$ solution in a humid chamber for 3 h at 56°C, washed 4 times in distilled water and air dried. Stained slides were observed under a light microscope to select suitably spread cells. Once selected, plastic (Falcon film) circles were cut out with a diamond tap and transferred onto grids and examined in a JEM 100B electron microscope.

Immunofluorescence. Poly-L-lysine-coated glass was used for immunostaining. The slides were placed in phosphate-buffered saline (PBS) and incubated overnight at 4°C with the following primary antibodies diluted in antibody dilution buffer (ADB: 3% bovine serum albumin - BSA, 0.05% Triton X-100 in PBS): rabbit anti-SCP3 1:200 (Abcam, Ab15093), human anti-centromere antibodies, ACA 1:200 (Anti-

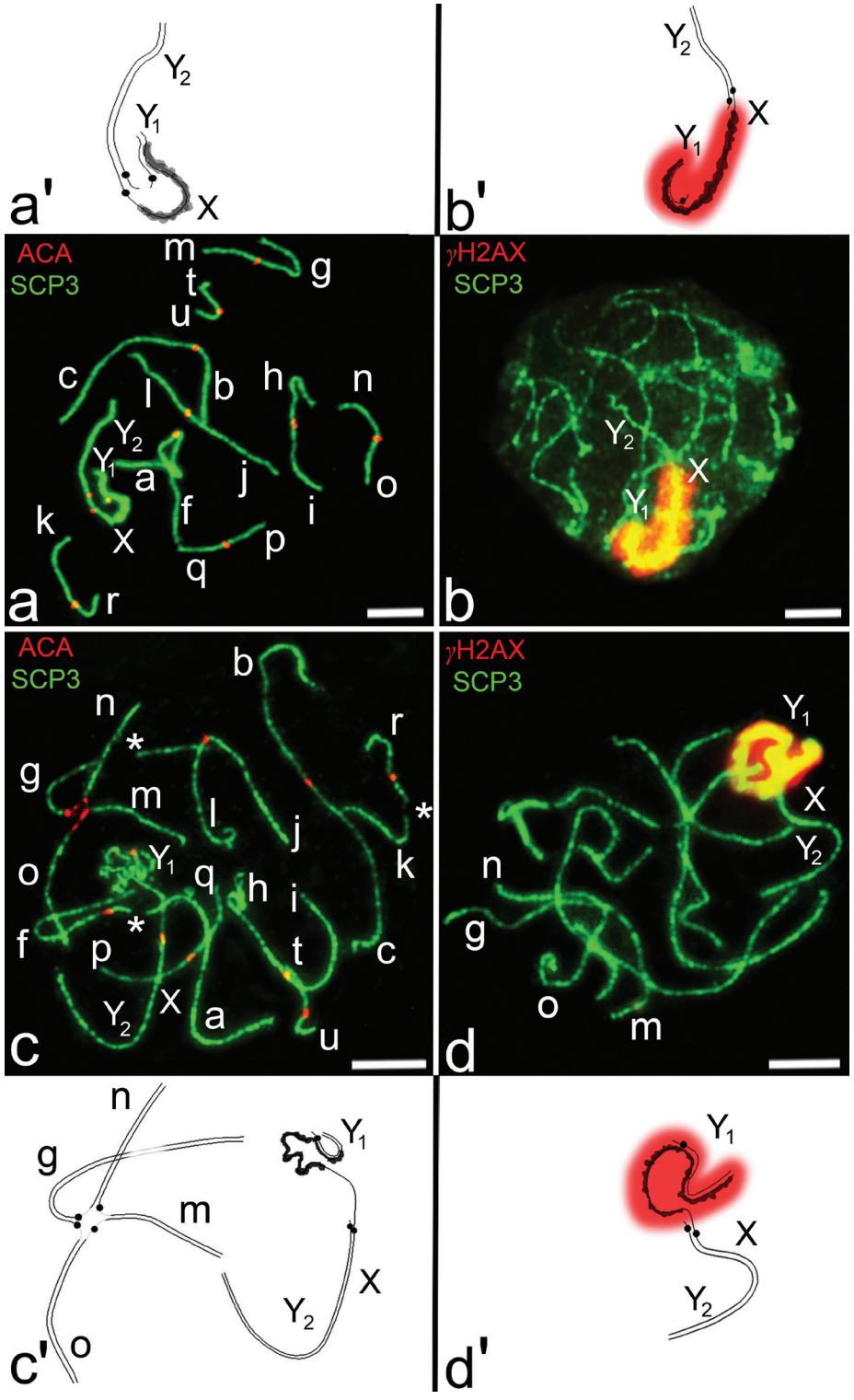
body Incorporated, 15-235) and mouse anti-phospho-histone γH2AX 1:500 (Abcam, Ab26350). After rinsing in PBS (3 times for 10 min), the slides were incubated with appropriate secondary antibodies diluted 1:800 in PBS: goat anti-rabbit Alexa Fluore 488 conjugated antibodies, goat anti-human Alexa Fluore 546 conjugated antibodies and FITC-conjugated horse anti-mouse IgG (all Abcam) at 37°C for 90 min. After a final rinse in PBS, the slides were mounted in Vectashield with DAPI (Vector Laboratories). Slides were analyzed in an Axioimager D1 microscope CHROMA filter sets (Carl Zeiss, Jena, Germany) equipped with an Axiocam HRm CCD camera (Carl Zeiss), and image-processing AxioVision Release 4.6.3. software (Carl Zeiss, Germany). Images were processed using Adobe Photoshop CS3 Extended.

Results

Karyotypes. Three of the eight karyotyped shrews were complex heterozygotes, i.e. F1 hybrids. They showed the expected arm combinations of Rb metacentrics - *go/gm/mn/no, hi, jl, kr, pq.* Five other shrews were homozygotes with Moscow race karyotype (*gm, hi, jl, kr, no, pq*). Hybrid individuals and homozygotes of the pure race had 2n=21, NF=40, XY_1Y_2. Only two hybrids and one homozygote were subject to comparative SC analysis.

SC analysis of a homozygote of the common shrew. Immunocytochemical analysis of SCs in pachytene spermatocytes of the homozygote revealed nine SC bivalents (*af*, *bc*, *jl*, *hi*, *gm*, *no*, *kr*, *pq*, *tu*) and the sex trivalent (XY_1Y_2), as expected from the G-banded karyotype of the Moscow race. Centromeres of *hi* SC bivalent and centromeres in the sex trivalent were not aligned. The sex trivalent exhibited irregular thickenings of the "true" arm of the X chromosome. The autosomal arm of the X chromosome formed a typical SC (Fig. 1a, a'). γH2AX covered only the synaptic region of the X and

Figure 1. a–d Synaptonemal complexes of homozygotes and complex heterozygotes of the common shrew. Immunostaining with antibodies against axial elements of SC - SCP3 (green), polyclonal antibodies to centromeric protein ACA (red) and antibodies to γH2AX (red) marking chromosome asynaptic regions. Bar = 5 μm **a, b** SCs from spermatocyte pachytene nuclei (the Moscow race) **a** Nine SC bivalents (*af*, *bc*, *jl*, *hi*, *gm*, *no*, *kr*, *pq*, *tu*) and sex trivalent XY_1Y_2. Sex trivalent contains irregular thickening of the "true" arm of X-chromosome (scheme **a'**). The autosomal arm of the X-chromosome forms a typical SC. Centromeres within *hi* bivalent and XY_1Y_2 trivalent are displaced relative to each other **b** Anti-γH2AX antibodies recognize chromatin in the synaptic zone of X and Y_1 chromosomes and unsynapsed thickened region of the "true" arm of X-chromosome (scheme **b'**) **c, d** SCs from spermatocyte pachytene nuclei obtained from Moscow-Neroosa hybrids **c** Seven SC bivalents (*af*, *bc*, *jl*, *hi*, *kr*, *pq*, *tu*), sex trivalent XY_1Y_2 and SC tetravalent (*g/o/n/m*) were revealed in spermatocyte nuclei of complex heterozygotes. Gaps were detected in SC bivalents *af*, *kr* and in *g* arm of SC-tetravalent (indicated with asterisks). Gaps were also detected in pericentromeric regions of all metacentrics of the SC tetravalent (scheme **c'**). *af* SC bivalent is associated with sex trivalent; **d** Anti-γH2AX antibodies identify chromatin in the synaptic region of X and Y_1 chromosomes and asynaptic thickening of the "true" arm of the X-chromosome (scheme **d'**), as for common shrew spermatocytes from Moscow race (Fig. 2b). One of the SC bivalents is associated with the true part of sex trivalent. The SC tetravalent is usually associated with one or two autosomes (**c, d**).


a'
Y2
Y1
X
b'
Y2
X
Y1
ACA
SCP3
m
t
u
g
c
l
b
h
n
Y2
Y1
a
j
o
k
f
i
X
p
q
r
a
γH2AX
SCP3
Y2
X
Y1
b
ACA
SCP3
b
n
*
r
g
m
l
j
*
k
o
Y1
q
h
i
f
p
*
t
c
Y2
X
a
u
c
γH2AX
SCP3
Y1
X
Y2
n
g
o
m
d
n
g
Y1
m
X
Y2
o
c'
Y1
X
Y2
d'

the Y_1 chromosomes and the thickened part of the X chromosome. The autosomal arm of the X chromosome is not involved in inactivation (Fig. 1b, b').

SC analysis of complex heterozygotes of the common shrew. As expected, seven SC bivalents (*af*, *bc*, *jl*, *hi*, *kr*, *pq*, *tu*), an SC tetravalent (*g*/*o*/*n*/*m*) and the sex trivalent XY_1Y_2 were detected in spermatocyte nuclei at pachytene stage (Fig. 1c, c', 2). According to the previously elaborated classification, the SC tetravalent represents a closed SC multivalent which was formed due to monobrachial homology (Matveevsky and Kolomiets 2011). Arms *af*, *kr* of SC bivalents, the *g* arm and pericentromeric regions of the SC tetravalent contain gaps (Fig. 1c). Sex trivalents were recurrently located at the periphery of the pachytene nuclei of spermatocytes. The "true" X arm of the sex trivalent was irregularly thickened and covered with γH2AX (Fig. 1d, d').

Suspension of testis cells. There are spermatocytes and active spermatozoa in testis cell suspension from common shrews of both Moscow race and hybrids (Fig. 3). Chromosome spreads also contained a significant amount of spermatozoa (Fig. 3 a', b').

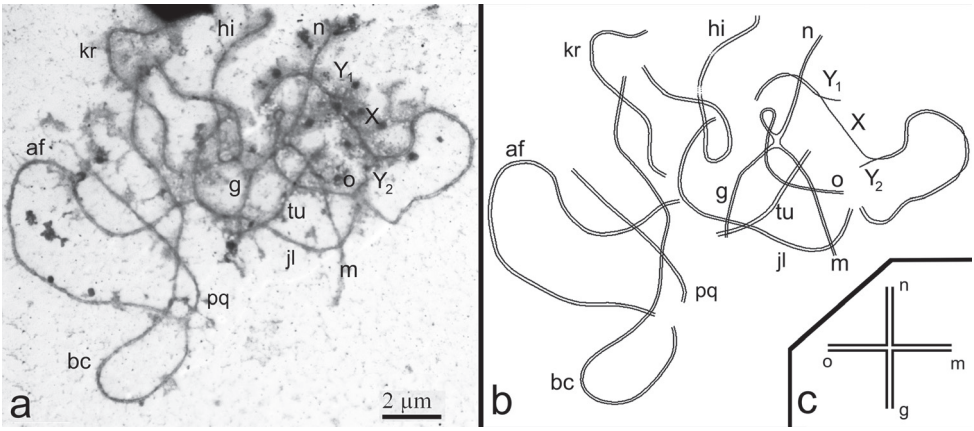


Figure 2. a–d A pachytene spermatocyte of the Moscow-Neroosa hybrid. **a** An electron micrograph. Seven SC bivalents (*af*, *bc*, *jl*, *hi*, *kr*, *pq*, *tu*), the sex trivalent XY_1Y_2 and the SC tetravalent (*g*/*o*/*n*/*m*) are detected. Closed SC tetravalent is composed of four monobrachial homologous metacentrics *go*, *on*, *nm*, *mg*. SC tetravalent is associated with two autosomes and sex trivalent. Bar = 2 µm **b** A scheme of chromosome synapsis on the basis of Fig. 2a **c** A scheme of SC tetravalent.

Discussion

Hybrid zones of *S. araneus* represent unique natural laboratories for studying Robertsonian chromosomal polymorphism. Complex cytogenetic studies have been carried out in 17 known chromosomal hybrid zones; however only seven of them have been subjected to analysis of early stages of meiosis including synaptonemal complexes analysis (see Table 1). Such studies provide information about the peculiarities of chromosomal synapsis and separation of multivalents in meiosis, which determine a hybrid's sterility/fertility. The latter is important for estimation of reproductive isolation level between different chromosomal races.

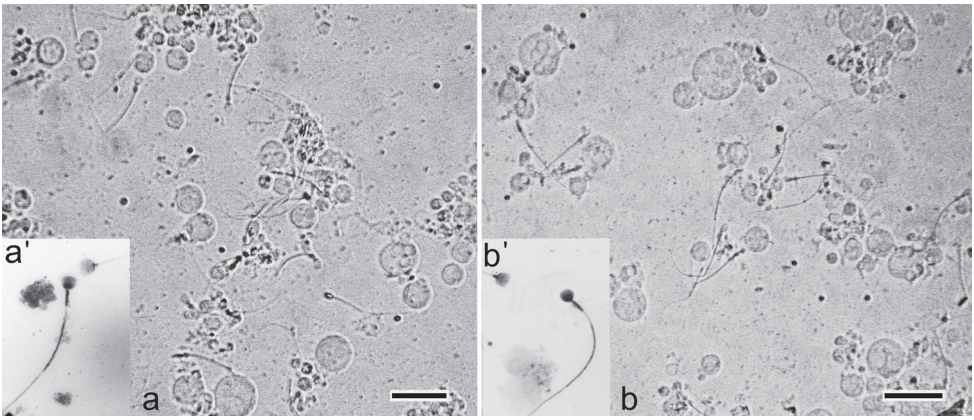


Figure 3. a–b Cell suspension of common shrew testis **a** – homozygote (the Moscow race) **b** – complex heterozygote from interracial hybrid zone **a', b'** Inverted image of spermatozoa (non-specific binding of anti-SCP3 antibodies after immunocytochemistry). Bar = 20 μm.

Table 1. SC analysis in chromosomal hybrid zones of the common shrew.

Hybrid zone	Examined karyotypic categories	Detected SC-configuration	Reference
Oxford/ Hermitage	SH (chain-of-three) *(k/q), (n/o), (p/r)*	SC trivalents	Wallace and Searle 1990
Oxford/ Wirral	SH (chain-of-three) *(k/q), (n/o), (k/o), (j/l)*	SC trivalents	Borodin et al. 2008
Abisko/ Sidensjö	CH (chain-of-four) *i/ih/hn/n*	SC tetravalent	Narain and Fredga 1998
Aberdeen/ Oxford	CH (chain-of-seven) *r/rp/pn/no/ok/kq/q*	SC chain with 7 elements	Mercer et al. 1992
Novosibirsk/ Tomsk	CH (chain-of-eight) + (chain-of-three) *o/og/gk/ki/ih/hn/nm/m, q/r*	SC chain with 8 elements and SC trivalent	Karamysheva et al. 2007
Moscow/ Seliger	CH (chain-of-eleven) *g/gm/mh/hi/ik/kr/rp/pq/qn/no/o*	SC chain with 11 elements	Pavlova et al. 2008
Uppsala/ Hällefors	CH (ring-of-four) *qp/pk/ko/oq*	SC tetravalent	Narain and Fredga 1997
Moscow/ Neroosa	CH (ring-of-four) *og/gm/mn/no*	SC tetravalent	this study

SH – simple heterozygotes, CH – complex heterozygotes

The model of chromosomal speciation by monobrachial centric fusion has been proposed by Baker and Bickham (1986). Fixation of metacentric chromosomes with monobrachial (single-arm) homology formed as a result of independent fusion of acrocentric chromosomes can entail reproductive isolation of population and further speciation due to the accumulation of genetic differences (Capanna 1982, Baker and Bickham 1986). It is considered that such a mechanism of speciation occurs among some mammal species: lemur genus *Eulemur* Simons et Rumpler, 1989 (Djlelati et al.

1997, Rumpler 2004), bat genus *Rhogeessa* H. Allen, 1866 (Baker et al. 1985), mole vole of the *Ellobius tancrei* (Bakloushinskya et al. 2010), beaver genus *Castor* Linnaeus, 1758 (Ward et al. 1991), mouse of the *Mus musculus domesticus* (Capanna et al. 1976), rat genus *Rattus* Fischer de Waldheim, 1803 (Baverstock et al. 1983). Searle (1988) suggested that monobrachial fusions may contribute to speciation in *Sorex araneus*.

It was assumed that interracial hybrids of the common shrew (complex heterozygotes with multiple Rb rearrangements) had either significantly reduced fertility or were completely sterile (Searle 1988, 1993, Aniskin and Lukianova 1989). Mice and mole voles that differed in several Rb translocations, exhibited reduced fertility too (Capanna 1975, Lyapunova et al. 1990, Hauffe and Searle 1998, Bakloushinskaya et al. 2010). Furthermore, reduced fertility and presence of aneuploid cells were revealed in heterozygotes from different hybrid zones of house mouse chromosomal races that varied in monobrachial homologous metacentrics (Said et al. 1993, Nunes et al. 2011). The fertility of hybrids most likely depends on the amount of monobrachial homologous metacentrics. Indeed, complex heterozygotes of *S. araneus* from the contact zone of Oxford and Hermitage races had an increased content of defective testicular tubes and testis with reduced weight, whereas simple heterozygotes were similar to homozygotes (Garagna et al. 1989). However, in the common shrew, even extremely long meiotic chain configurations may not necessarily lead to complete sterility (Mercer et al. 1982). For example, hybrids from a contact zone of Moscow and Seliger races that exhibited the most diverse pattern of monobrachial homologous metacentrics with an additional WART translocation had mature spermatozoa (Pavlova et al. 2008).

Previous studies have also demonstrated that association of autosomes and complex SC configurations with sex chromosomes in meiotic prophase I could cause reduction of fertility or even complete sterility (Forejt et al. 1981, Burgoyne and Baker 1984). It should be noted that, unlike SC trivalents, complex SC configurations are often associated with autosomes and sex trivalents (Narain and Fredga 1997, Pavlova et al. 2008). We also revealed that sometimes the SC tetravalent interacted with the sex trivalent in the complex heterozygotes that we examined. In previous works similar contact sites (or physically interactions) of autosomes, SC multivalents and sex bivalents were interpreted as associations (Mercer et al. 1982, Narain and Fredga 1997).

To reveal the signs of defects in spermatogenesis in our specimens, we studied the dynamics of meiotic prophase I focusing on the sex trivalent. Normally, sex chromosomes of male mammals contain a short SC in pseudoautosomal region and long unpaired axes in meiotic prophase I. Also, sex chromosomes often move to the periphery of pachytene nuclei and undergo MSCI (*meiotic sex chromosome inactivation*), which is required for successful progression of meiosis (Forejt 1984, Burgoyne et al. 2009). We used SCP3 antibodies to identify the SC axial elements and γH2AX antibodies to mark chromosome asynaptic regions. This marker was revealed in chromosome asynaptic regions starting from leptotene and up to late diplotene in cases of incomplete synapsis (Turner et al. 2006).

We found that the behavior of the sex trivalent in homozygotes (Moscow race) was similar to that of sex chromosomes in meiotic prophase I in other mammals. How-

ever, in complex heterozygotes, the sex trivalent interacted with autosomes in some prophase nuclei, which was typical of hybrids and heterozygotes with chromosomal rearrangements and reduced fertility (Forejt et al. 1981). Thus, we do not exclude a possibility of partial loss of spermatocytes due to this condition.

Formation of complex SC configurations is known to be associated with a high degree of asynapsis. In such cases, chromosome asynaptic regions undergo transcriptional inactivation MSUC (*meiotic silencing of unsynapsed chromatin*), which in its turn results in meiotic arrest and reduction of fertility (Homolka et al. 2007, Mahadevaiah et al. 2008). Nonetheless, no MSUC signs were detected in the hybrids of *S. araneus*. In the gap regions (SC tetravalent, *af*, *kr* SC bivalents), γH2AX was not detected. Most probably, gaps in pericentromeric regions of the SC tetravalent do not reflect asynaptic regions, but may result from the extension of chromosome axial elements due to the alteration of nucleus organization and retention of telomere links with nuclear envelope. Similar trends in tetravalent dynamics were revealed in the progeny of radiation-exposed male mice (Kolomiets et al. 1992). Closed SC multivalents associate with the sex bivalent to a lesser extent and therefore do not cause meiotic failures.

Probably, the four metacentrics that form SC tetravalent in interracial Moscow-Neroosa hybrids undergo successful separation, spermatocytes are not arrested (or are arrested partially) and balanced gametes are formed in the end. This is also supported by the presence of numerous spermatozoa in hybrid testis cell suspensions. Further studies are needed to measure the level of aneuploidy.

Our data conform to the results of other authors. For example, no defects of sex body formation were detected in most spermatocyte nuclei of mice that were heterozygous for eight Rb translocations, which indicated moderate activity of pachytene arrest (Manterola et al. 2009). Association of the SC trivalent carrying asynaptic regions with the XY bivalent in early-middle pachytene, which also did not result in the reduction of fertility, was revealed with immunocytochemical methods in laboratory mice with a single translocation (Saferali et al. 2010). Apparently, in case of Rb translocations the reduced efficiency of checkpoint in pachytene determines the possibility of Rb metacentric circulation in natural populations and their role in karyotype evolution (Matveevsky and Kolomiets 2011).

In our study, centromeres of homologues in the SC bivalent formed between two Rb metacentrics (*hi*) were not aligned. We assume that this pattern might result from different mechanisms of Rb metacentric formation in the past. One of the ancestors might have retained centromere of *h* chromosome after formation of Rb metacentric, while another ancestor might have retained centromere of *i* chromosome. Previous works reported a presence of two centromeric foci in other Rb metacentrics (Borodin et al. 2008). We suggest that in case of extensive chromosomal variability of *S. araneus,* centromere polymorphism of Rb metacentrics might be often observed in different populations.

Our synaptonemal complex results suggest regularity in formation of the ring-of-four configuration produced by hybrids between the Moscow and the Neroosa chromosome races of *S. araneus*. This relates well to the previous findings that chro-

mosome races differing by monobrachial homology in *S. araneus* does not lead to complete sterility in hybrids. In particular, our immunocytochemical demonstration of an absence of asynapsis in the ring-of-four configuration relates well to the production of sperm in such hybrids.

Acknowledgments

We would like to thank Dr Nina Bulatova for discussion on the structure of the manuscript and valuable comments in karyology. We are especially grateful to Prof Jeremy B. Searle for valuable comments on the manuscript and the English. This work was supported by President Grant for Russian Distinguished Young Scientists MK-2500.2011.4 (for SP) and research grants of the Russian Foundation for Basic Research № 12-04-31425, 12-04-31200.

References

Aniskin VM, Lukianova IV (1989) New chromosome race and analysis of the hybrid zone between two karyomorphs of *Sorex araneus* (Insectivora, Soricidae). Proceedings of the USSR Academy of Sciences 309: 1260–1262. [In Russian]

Baker RJ, Bickham JW (1986) Speciation by Monobrachial Centric Fusions. Proceedings of the National Academy of Sciences of USA 83: 8245–8248. doi: 10.1073/pnas.83.21.8245

Baker RJ, Bickham JW, Arnold ML (1985) Chromosomal evolution in *Rhogeessa* (Chiroptera: Vespertilionidae): Possible speciation by centric fusions. Evolution 39: 233–243. doi: 10.2307/2408359

Bakloushinskya IYu, Romanenko SA, Graphodatsky AS, Matveevsky SN, Lyapunova EA, Kolomiets OL (2010) The role of chromosome rearrangements in the evolution of mole voles of the genus *Ellobius* (Rodentia, Mammalia). Russian Journal of Genetics 46: 1143–1145. doi: 10.1134/S1022795410090346

Baverstock PR, Gelder M, Jahnke A (1983) Chromosome evolution in Australian Rattus - G-banding and hybrid meiosis. Genetica 60: 93–103. doi: 10.1007/BF00127495

Borodin PM, Karamysheva TV, Belonogova NM, Torgasheva AA, Rubtsov NB, Searle JB (2008) Recombination map of the common shrew, *Sorex araneus* (Eulipotyphla, Mammalia). Genetics 178(2): 621–32. doi: 10.1534/genetics.107.079665

Bulatova N, Searle JB, Nadjafova RS, Pavlova SV, Bystrakova NV (2009) Field protocols for the genomic era. Comparative Cytogenetics 3: 57–62. doi: 10.3897/compcytogen.v3i1.9

Bulatova N, Jones RM, White TA, Shchipanov NA, Pavlova SV, Searle JB (2011) Natural hybridization between extremely divergent chromosomal races of the common shrew (*Sorex araneus*, Soricidae, Soricomorpha): hybrid zone in European Russia. Journal of Evolutionary Biology 24(3): 573–586. doi: 10.1111/j.1420-9101.2010.02191.x

Burgoyne PS, Baker TG (1984) Meiotic pairing and gametogenic failure. Symposia of the Society for Experimental Biology 38: 349-362.

Burgoyne PS, Mahadevaiah SK, Turner JM (2009) The consequences of asynapsis for mammalian meiosis. Nature Reviews Genetics 10: 207–216. doi: 10.1038/nrg2505

Capanna E (1975) Gametic aneuploidy in mouse hybrids. Chromosomes Today 5: 83–89.

Capanna E (1982) Robertsonian Numerical variation in Animal Speciation: *Mus musculus* an emblematic model. In: Barigozzi C (ed) Mechanisms of Speciation, Alan R. Liss, New York, 155-177.

Capanna E, Gropp A, Winking H, Noack G, Civitelli MV (1976) Robertsonian metacentrics in the mouse. Chromosoma 58: 341–353. doi: 10.1007/BF00292842

Djlelati R, Brun B, Rumpler Y (1997) Meiotic study of hybrids in the genus Eulemur and taxonomic considerations. American Journal of Primatology 42(3): 235–245. doi: 10.1002/(SICI)1098-2345(1997)42:3<235::AID-AJP6>3.0.CO;2-Y

Ford CE, Hamerton JL (1956) A colchicine, hypotonic citrate, squash sequence for mammalian chromosomes. Stain Technology 31: 247–251.

Forejt J (1984) X-inactivation and its role in male sterility. Chromosomes Today 8: 17-22.

Forejt J, Gregorov S, Goetz P (1981) XY pair associates with the synaptonemal complex of autosomal male-sterile translocations in pachytene spermatocytes of the mouse (*Mus musculus*). Chromosoma 82: 41–53. doi: 10.1007/BF00285748

Fredga K (1970) Unusual sex chromosome inheritance in mammals. Philosophical Transactions of the Royal Society of London. Biological Sciences 259: 15–36. doi: 10.1098/rstb.1970.0042

Garagna S, Zuccotti M, Searle JB, Redi CA, Wilkinson PJ (1989) Spermatogenesis in heterozygotes for Robertsonian rearrangements from natural population of the common shrew *Sorex araneus*. Journal of Reproduction and Fertility 87: 431–438. doi: 10.1530/jrf.0.0870431

Gropp A, Winking H, Zech L, Müller HJ (1972) Robertsonian chromosomal variation of metacentric chromosomes in feral mice. Chromosoma 39: 265–288. doi: 10.1007/BF00290787

Gropp A, Winking H (1981) Robertsonian translocations: Cytology, meiosis, segregation patterns and biological consequences of heterozygosity. Symposia of the Zoological Society of London 47: 141–181.

Hausser J, Fedyk S, Fredga K, Searle JB, Volobouev V, Wójcik J, Zima J (1994) Definition and nomenclature of the chromosome races of *S. araneus*. Folia Zoologica 43(1): 1–9.

Hauffe HC, Searle JB (1998) Chromosomal heterozygosity and fertility in house mice (*Mus musculus domesticus*) from northern Italy. Genetics 150: 1143–1154.

Homolka D, Ivanek R, Capkova J, Jansa P, Forejt J (2007) Chromosomal rearrangement interferes with meiotic X chromosome inactivation. Genome Research 17(10): 1431–1437. doi: 10.1101/gr.6520107

Jadwiszczak KA, Banaszek A (2006) Fertility in the male common shrews, Sorex araneus, from the extremely narrow hybrid zone between chromosome races. Mammalian Biology 71: 257–267. doi: 10.1016/j.mambio.2006.02.004

Jotterand M (1972) Le polymorphisme chromosomique des *Mus* (Leggadas) africains: cytogénétique, zoogéographie, évolution. Revue Suisse de Zoologie 79: 287–359.

Karamysheva TV, Belonogova NM, Rodionova MI, Rubtsov NB, Polyakov AV, Searle JB, Borodin PM (2007) Temporal and spatial distribution of Rad51 protein in spermatocytes of the common shrew *Sorex araneus* L. (Soricidae, Eulipotyphla). Russian Journal of Theriology 6(1): 15–19.

King M (1993) Species Evolution: The Role of Chromosomal Change. Cambridge, 360 pp.

Kolomiets OL, Matveevsky SN, Bakloushinskaya IYu (2010) Sexual dimorphism in prophase I of meiosis in mole vole (*Ellobius talpinus* Pallas) with isomorphic (XX) chromosomes in males and females. Comparative Cytogenetics 4(1): 55–66. doi: 10.3897/compcytogen.v4i1.25

Kolomiets OL, Mazurova TF, Pomerantseva MD, Chekhovich AV, Bogdanov YuF (1992) Electron microscopic analysis of the synaptonemal complexes in male laboratory mice exposed in the Chernobyl Region during embryogenesis. Russian Journal of Genetics 28(9): 120–127. [In Russian]

Král B, Radjabli SI (1974) Banding patterns and Robertsonian fusions in the western Siberian population of *Sorex araneus* (Insectivora, Soricidae). Folia Zoologica 23: 217–227.

Lyapunova EA, Vorontsov NN, Korobitsina KV, Ivanitskaya EYu, Borisov YuM, Yakimenko L V, Dovgal VY (1980) A Robertsonian fan in *Ellobius talpinus*. Genetica 52/53: 239–247. doi: 10.1007/BF00121833

Lyapunova EA, Bakloushinskaya IYu, Kolomiets OL, Mazurova TF (1990) Analysis of fertility of hybrids of multi chromosomal forms in mole voles of the superspecies *Ellobius tancrei* differing in a single pair of Robertsonian metacentrics. Proceedings of the USSR Academy of Sciences 310(3): 721–723. [In Russian]

Mahadevaiah SK, Bourc'His D, de Rooij DG, Bestor TH, Turner JM, Burgoyne PS (2008) Extensive meiotic asynapsis in mice antagonises meiotic silencing of unsynapsed chromatin and consequently disrupts meiotic sex chromosome inactivation. Journal of Cell Biology 182:263–276. doi: 10.1083/jcb.200710195

Manterola M, Page J, Vasco C, Berríos S, Parra M, Viera A, Rufas J, Zuccotti M, Garagna S, Fernández-Donoso R (2009) A high incidence of meiotic silencing of unsynapsed chromatin is not associated with substantial pachytene loss in heterozygous male mice carrying multiple simple Robertsonian translocations. PLoS Genetics 8: 1–14. doi: 10.1371/journal.pgen.1000625

Matthey R (1970) The "Robertsonian fan" in African *Mus* (*Leggada*) of the group *minutoides-musculoides*). Revue Suisse de Zoologie 77: 625–629.

Matveevsky SN, Kolomiets OL (2011) Types of synaptonemal complex chains in heterozygotes for multiple Robertsonian translocations. Factors of Experimental Evolution of Organisms 10: 42–47. [In Russian]

Mercer SJ, Wallace BMN, Searle JB (1992) Male common shrews (*Sorex araneus*) with long meiotic chain configurations can be fertile: implications for chromosomal models of speciation. Cytogenetic and Cell Genetics 60: 68–73. doi: 10.1159/000133298

Narain Y, Fredga K (1997) Meiosis and fertility in common shrew, *Sorex araneus*, from a chromosomal hybrid zone in central Sweden. Cytogenetic and Cell Genetics 78: 253–259. doi: 10.1159/000134668

Narain Y, Fredga K (1998) Spermatogenesis in common shrews, *Sorex araneus*, from a hybrid one with extensive Robertsonian polymorphism. Cytogenetic and Cell Genetics 80: 158–164. doi: 10.1159/000014973

Navarro J, Vidal F, Quitart M (1981) A method for the sequential study of SC by light and electron microscopy. Human Genetics 59: 419–423. doi: 10.1007/BF00295483

Nunes AC, Catalan J, Lopez J, M da Graça Ramalhinho, M da Luz Mathias, Britton-Davidian J (2011) Fertility assessment in hybrids between monobrachially homologous Rb races of the house mouse from the island of Madeira: implications for modes of chromosomal evolution. Heredity 106(2): 348–356. doi: 10.1038/hdy.2010.74

Orlov VN, Borisov YuM, Cherepanova EV, Grigor'eva OO, Shestak AG, Sycheva VB (2012) Narrow hybrid zone between Moscow and Western Dvina chromosomal races and specific features of population isolation in common shrew *Sorex araneus* (Mammalia). Russian Journal of Genetics 48(1): 70–78. doi: 10.1134/S1022795412010152

Pack SD, Borodin PM, Serov OL, Searle JB (1993) The X-autosome translocation in the common shrew (*Sorex araneus* L.): late replication in female somatic cells and pairing in male meiosis. Chromosoma 102: 355–360. doi: 10.1007/BF00661279

Pavlova SV, Kolomiets OL, Bulatova N, Searle JB (2008) Demonstration of a WART in a hybrid zone of the common shrew (*Sorex araneus* Linnaeus, 1758). Comparative Cytogenetics 2(2): 115–120.

Pavlova SV, Matveevsky SN, Acaeva MM, Kolomiets OL, Bulatova N (2012) Can chromosomal hybrid zones promote speciation in the common shrew *Sorex araneus*: what does the study of meiosis? International Conference "Chromosome 2012". Novosibirsk, September 2–7, 2012. [in press]

Polyakov A, White Th, Jones RM, Borodin PM, Searle JB (2011) Natural hybridization between extremely divergent chromosomal races of the common shrew (Sorex araneus, Soricidae, Soricomorpha): hybrid zone in Siberia. Journal of Evolutionary Biology 24: 1393–1402. doi: 10.1111/j.1420-9101.2011.02266.x

Robertson WRB (1916) Chromosome studies. I. Taxonomic relationships shown in the chromosomes of Tettigidae and Acrididae: V-shaped chromosomes and their significance in Acrididae, Locustidae, and Gryllidae: chromosomes and variation. Journal of Morphology 27: 179–181. doi: 10.1002/jmor.1050270202

Rumpler Y (2004) Complementary approaches of cytogenetics and molecular biology to the taxonomy and study of speciation processes in Lemurs. Evolutionary Anthropolgy 13: 67–78. doi: 10.1002/evan.20010

Saferali A, Berlivet S, Schimenti J, Bartolomei MS, Taketo T, Naumova AK (2010) Defective imprint resetting in carriers of Robertsonian translocation Rb (8.12). Mammalian Genome 21: 377–387. doi: 10.1007/s00335-010-9271-9

Said K, Saad A, Auffray J-C, Britton-Davidian J (1993) Fertility estimates in the Tunisian all-acrocentric and Robertsonian populations of the house mouse and their chromosomal hybrids. Heredity 71:532–538. doi: 10.1038/hdy.1993.172

Searle JB (1988) Selection and Robertsonian variation in nature: the case of the common shrew. In: Daniel A (Ed.) The Cytogenetics of Mammalian Autosomal Rearrangements. New York, 507–531.

Searle JB (1993) Chromosomal hybrid zones in eutherian mammals. In: Harrison RG (Ed.) Hybrid Zones and the Evolutionary Process. Oxford University Press, New York, 309–353.

Searle JB, Fedyk S, Fredga K, Hausser J, Volobouev VT (1991) Nomenclature for the chromosomes of the common shrew (*Sorex araneus*). Mémoires de la Société vaudoise des sciences naturelles 19: 13–22.

Searle JB, Wójcik JM (1998) Chromosomal evolution: the case of *Sorex araneus*. In: Wójcik JM, Wolsan M (Eds) Evolution of Shrews. Białowieża, 219–268.

Sharman GB (1956) Chromosomes of the common shrew. Nature 177: 941–942. doi: 10.1038/177941a0

Tembotov AK, Khatukhov AM, Ivanov VG, Grigorieva GI (1976) An ecological and geographical aspect of the evolution of the pine vole in the Caucasus. In: Fauna, ecology and protection of North Caucasus animals, Nalchik, 3: 1–35. [In Russian]

Turner JMA, Mahadevaiah SK, Ellis PJI, Mitchell MJ, Burgoyne PS (2006) Pachytene asynapsis drives meiotic sex chromosome inactivation and leads to substantial postmeiotic repression in spermatids. Developmental Cell 10(4): 521–529. doi: 10.1016/j.devcel.2006.02.009

Volobouev V, Viegas-Péquignot E, Petter F, Gautun JC, Sicard B, Dutrillaux B (1988) Complex chromosomal polymorphism in *Gerbillus nigeriae* (Rodentia, Gerbillidae). Journal of Mammalia 69: 131–134. doi: 10.2307/1381757

Vorontsov NN (1960) Species of Palaearctic hamsters (Cricetinae, Rodentia) in *statu nascendi*. Proceedings of the USSR Academy of Sciences 132: 1065–1069. [In Russian]

Wallace BMN, Searle JB (1990) Synaptonemal complex studies of the common shrew (*Sorex araneus*). Comparison of Robertsonian heterozygotes and homozygotes by light microscopy. Heredity 65: 359–367. doi: 10.1038/hdy.1990.105

Ward OG, Graphodatsky AS, Wurster-Hill DH, Ermina VR, Park JP, Yu Q (1991) Cytogenetics of beavers: a case of speciation by monobrachial centric fusions. Genome 34: 324–328. doi: 10.1139/g91-053

White MJD (1978) Modes of speciation. San Francisco, California, 455 pp.

White MJD (1982) Rectangularity, speciation and chromosome architecture. In: Barigozzi C (ed) Mechanisms of Speciation, Alan R. Liss, New York, 75–103.

White TA, Bordewich M, Searle JB (2010) A network approach to study karyotypic evolution: the chromosomal races of the common shrew (*Sorex araneus*) and house mouse (*Mus musculus*) as model systems. Systematic Biology 59: 262–276. doi: 10.1093/sysbio/syq004

Zima J, Lukacova L, Macholan M (1998) Chromosomal evolution in shrews. In: Wójcik JM, Wolsan M (Eds) Evolution of shrews. Białowieża, 175–218.

CompCytogen 6(3): 315–321 (2012)
doi: 10.3897/CompCytogen.v6i3.2667
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Localization of 18S ribosomal genes in suckermouth armoured catfishes Loricariidae (Teleostei, Siluriformes) with discussion on the Ag-NOR evolution

Anderson Luis Alves[1,2], Rafael Splendore de Borba[2], Allan Pierre Bonetti Pozzobon[2], Claudio Oliveira[3], Mauro Nirchio[4], Angel Granado[4], Fausto Foresti[3]

1 *Embrapa Pesca e Aquicultura (CNPASA), Palmas, Tocantins, Brazil Embrapa Pesca e Aquicultura (CNPASA), Palmas, Tocantins, Brazil* **2** *Laboratório de Citogenética, Univ Estadual Paulista "Julio de Mesquita Filho" - UNESP, Rio Claro, São Paulo State, Brazil* **3** *Departamento de Morfologia, Instituto de Biociências, Univ Estadual Paulista Julio de Mesquita Filho, Botucatu, São Paulo State, Brazil* **4** *Instituto Limnológico, Universidad de Oriente, Caicara del Orinoco, Estado Bolívar, Venezuela*

Corresponding author: *Anderson Luis Alves* (anderson.alves@embrapa.br)

Academic editor: *N. Golub* | Received 12 January 2012 | Accepted 11 September 2012 | Published 26 September 2012

Citation: Alves AL, Borba RS, Pozzobon APB, Oliveira C, Nirchio M, Granado A, Foresti F (2012) Localization of 18S ribosomal genes in suckermouth armoured catfishes Loricariidae (Teleostei, Siluriformes) with discussion on the Ag-NOR evolution. Comparative Cytogenetics 6(3): 315–321. doi: 10.3897/CompCytogen.v6i3.2667

Abstract

The family Loricariidae with about 690 species divided into six subfamilies, is one of the world's largest fish families. Cytogenetic studies conducted in the family showed that among 90 species analyzed the diploid number ranges from 2n=38 in *Ancistrus* sp. to 2n=96 in *Hemipsilichthys gobio* Luetken, 1874. In the present study, fluorescence *in situ* hybridization (FISH) was employed to determine the chromosomal localization of the 18S rDNA gene in four suckermouth armoured catfishes: *Kronichthys lacerta* (Nichols, 1919), *Pareiorhaphis splendens* (Bizerril, 1995), *Liposarcus multiradiatus* (Hancock, 1828) and *Hypostomus* prope *plecostomus* (Linnaeus, 1758). All species analyzed showed one chromosome pair with 18S rDNA sequences, as observed in the previous Ag-NORs analyses. The presence of size and numerical polymorphism was observed and discussed, with proposing a hypothesis of the Ag-NOR evolution in Loricariidae.

Keywords

Fish cytogenetics, fluorescent *in situ* hybridization, Loricariidae

Introduction

Fishes of the family Loricariidae are found in almost all South and Central America, from Costa Rica to Argentina and represent one of the world's largest fish families, with about 690 species described and about 300 undescribed (Reis et al. 2003). Recently this family has been divided into five subfamilies: Neoplecostominae, Hypoptopomatinae, Loricariinae, Hypostominae and a basal subfamily Delturinae (Armbruster 2004). About 100 Loricariidae species have been karyotyped so far (Oliveira and Gosztonyi 2000, Alves et al. 2006). The diploid chromosome number ranges from 2n=36 in *Rineloricaria latirostris* Boulenger, 1899 (Giuliano-Caetano 1998) to 2n=96 in *Hemipsilichthys gobio* Luetken, 1874 (Kavalco et al. 2004).

Ribosomal RNA genes are organized in fishes and in other groups as multiple copies of a repeated unit that consists of a transcribed zone with coding regions for the 18S, 5.8S and 28S rRNA genes, separated by internal and external transcribed spacers and surrounded by non-transcribed spacer sequences. The 18S rDNA gene probes by fluorescent *in situ* hybridization (FISH) have provided coincident markers with silver nitrate impregnation (AG-NOR) in nucleolar organizer region (Ag-NOR) in fish chromosomes (Paintner et al. 2002, Porto-Foresti et al. 2002, Fontana et al. 2003, Kavalco et al. 2005).

According to Foresti et al. (1981) the Ag-NORs with large size polymorphism and/or numeric polymorphism are frequent in Neotropical freshwater fishes. Thus, the detection of genes related with Ag-NORs is very important for the identification and characterization of these kinds of polymorphism (Wasko and Galetti Jr 2000). Ag-NORs size polymorphism is common in Loricariidae fishes, mainly in species with single Ag-NORs as in Hypoptopomatinae and Neoplecostominae, although, it can occur in species with multiple Ag-NORs as Hypostominae (Artoni and Bertollo 1996, Alves et al. 2003).

In the present study the localization of 18S rDNA genes was identified in four species for the first time. The results were compared to already published data on Ag-NOR, with the main objective of better understanding the changes involving ribosomal genes involved with Ag-NORs in Loricariidae fishes.

Material and methods

Cytogenetic analyses were performed on chromosome preparations obtained from four species collected in rivers from Brazil and Venezuela: *Kronichthys lacerta* (Nichols 1919), *Pareiorhaphis splendens* (Bizerril 1995), *Liposarcus multiradiatus* (Hancock 1828) and *Hypostomus* prope *plecostomus* (Linnaeus 1758) (Table 1). The specimens were analyzed by taxonomists that provided the species identification. The fishes were deposited in the fish collection of Laboratório de Biologia e Genética de Peixes (LBP), UNESP, Botucatu, SP, Brazil and in the Laboratório de Ictiologia, Museu de Ciências e Tecnologia, PUCRS (MCP), Porto Alegre, Brazil.

Chromosome preparations were obtained from kidney tissues using the technique described by Foresti et al. (1993) and were submitted to fluorescent *in situ* hybridization (FISH). Four probes employed in the Southern hybridizations techniques were used for FISH and they were labeled as follow: the double-strand probes (probes 1 and 4) were labeled by nick translation with biotin-14-dATP (Bionick labelling system-Gibco.BRL); the single-strand synthetic probes (probes 2 and 3) were labeled by random primer with biotin-14-dCTP (BioPrime DNA labeling system-Gibco.BRL). The metaphase chromosomes slides were incubated with RNAse (40 µg/ml) for 1.5 hour at 37°C. After the denaturation of chromosomal DNA in 70% formamide/2xSSC for 5 min at 70°C, 40µl of hybridization mixture (100ng of denatured probe, 50% formamide, 10mg/ml dextran sulfate, 2xSSC) was dropped on the slides and the hybridization was performed overnight at 37°C. Hybridization washes included 50% formamide in 2xSSC at 42°C and 2xSSC and 4xSSC at room temperature. Detection of hybridized probes was carried out with Avidin-FITC conjugate (Sigma) followed by two rounds of signal-amplification. After each step of amplification the slides were washed in a blocking buffer (1.26% NaHCO3, 0.018% sodium citrate, 0,0386% Triton/1% non-fat dried milk). Chromosomes were counterstained with Propidium Iodide, and the slides were mounted with Antifade (Vector).

Results and discussion

The karyotypes of the four species analyzed have been previously described in Alves (2005), Alves et al. (2005) and Alves et al. (2006), the diploid number and karyotype formulae with morphological classification in metacentric (m), submetacentric (sm) and subtelocentric (st) are presented in the Table 1.

The results showed that *Kronichthys lacerta* had only one signal of 18S rDNA in interstitial position in the long arm of the chromosome pair 21 (st) (Figure 1a), coinciding with a single Ag-NORs pattern presented by Alves et al. (2005). Beside the numerical polymorphism in this specie, the large size polymorphism of the 18S rDNA loci suggests a duplication of this gene in the active Ag-NOR chromosome.

Pareiohaphis splendens had two signal of 18S rDNA in interstitial position in the long arm of the chromosome pair 3 (m) (Figure 1b), coinciding with single Ag-NOR pattern presented by Alves et al. (2005). This species presented an evident variation in Ag-NORs size among homologous chromosomes that can be confirmed by the probe 18S rDNA. This structural polymorphism is common in the Loricariidae fishes (Artoni and Bertollo 1996, Alves et al. 2003, Alves et al. 2005).

In *Liposarcus multiradiatus* two signal of 18S rDNA in subterminal position in the long arm of the chromosome pair 10 (m) were detected (Figure 1c), coinciding with single Ag-NORs pattern presented by Alves et al. (2006). In this species a small size polymorphism of 18S rDNA occurs, although, the possible duplication or rearrangement events are not evident.

Table 1. A summary of the cytogenetic data available on the family Loricariidae with chromosomal localization ribosomal genes. 2n= diploid number; M= metacentric; SM= submetacentric; ST= subtelocentric; A= acrocentric.

Species	Locality	rDNA gene	2N	Karyotypic formulae	Reference
Neoplecostominae					
Kronichthys lacerta (Nichols, 1919)	Marumbi River, Brazil	18S	54	20M, 20SM, 14ST	Present study
Pareiorhaphis splendens (Bizerril, 1995)	Marumbi River, Brazil	18S	54	20M, 20SM, 14ST	Present study
Neoplecostomus microps Steindachner, 1877	Paraiba do Sul River, Brazil	18S	54	24M, 20SM, 10ST	Kavalco et al. (2005)
Delturinae					
* *Hemipsilicthys gobio* Luetken, 1874	Paraiba do Sul River, Brazil	18S	96	16M, 08SM, 72A	Kavalco et al. (2005)
Loricariinae					
Harttia loricarifomes Steindachner, 1877	Paraiba do Sul River, Brazil	18S	56	16M, 22SM, 10ST, 8A	Kavalco et al. (2005)
Hypostominae					
Liposarcus multiradiatus (Hancock, 1828)	Orinoco River, Venezuela	18S	52	22M, 18SM, 12ST	Present study
Hypostomus affinis Steindachner, 1886	Paraiba do Sul River, Brazil	18S	66	14M, 14SM, 12ST, 26A	Kavalco et al. (2005)
Hypostomus prope *plecostomus* (Linnaeus, 1758)	Orinoco River, Venezuela	18S	68	12M, 16SM, 12ST, 24A	Present study

Weak signals of 18S rDNA were presented in the *Hypostomus* prope *plecostomus*: two signals were observed in the short arm of the chromosome pair 16 (st) (Figure 1d). These signals are coincident with single Ag-NORs presented in this specie by Alves (2005). Different of the others species analyzed here, *Hypostomus* prope *plecostomus* does not presented size polymorphism of 18S rDNA, suggesting a conserved status of this character in this species.

There are few studies related to the identification of Ag-NOR regions through the technique of hybridization with 18S rDNA probes in Loricariidae fishes, the most data available are on *Hypostomus*. The hybridization techniques with the fluorochromes DAPI and CMA_3, were used to identified Ag-NOR regions of *Hypostomus nigromaculatus* (Schubart, 1964) (Rubert et al. 2008) and *H.* prope *wuchereri* (Günther, 1864) (Bitencourt et al. 2011). Artoni and Bertollo (1999) already used Mithramycin A (DAPI/MM) technique for observation of this region in *Hypostomus* sp., *Hypostomus* sp. B and *Hypostomus* sp. F. In a recent paper Mendes-Neto et al. (2011) identified the Ag-NOR regions in *H. regani* (Ihering, 1905) through the technique of hybridization with 18S rDNA probes, in all these works the species analysed showed single Ag-NOR in there chromosomes.

In conclusion, for Oliveira and Gosztonyi (2000) the condition of single Ag-NORs in subterminal position is the possible basal condition for the Siluriformes spe-

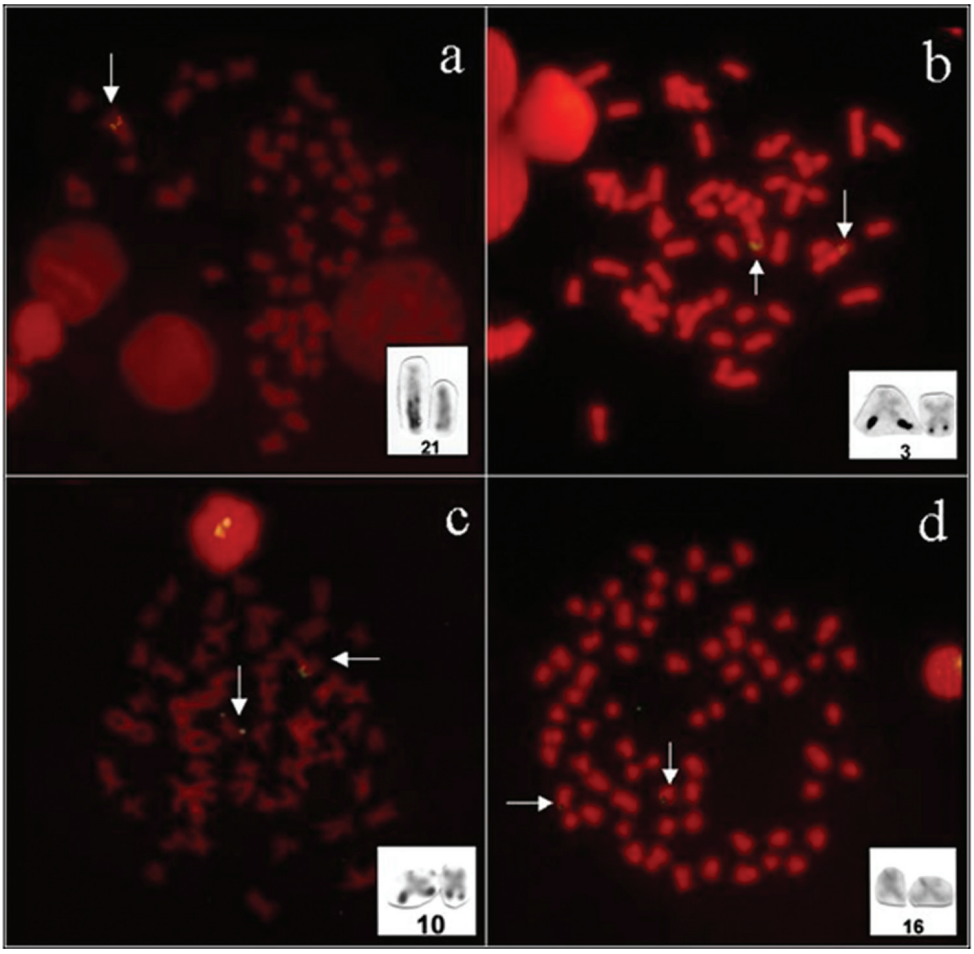


Figure 1. Fluorescent *in situ* hybridization with 18S rDNA probe in (**a**) *Kronichthys lacerta*, (**b**) *Pareiorhaphis splendens*, (**c**) *Liposarcus multiradiatus* and (**d**) *Hypostomus* prope *plecostomus*. Arrows indicate rDNA sites in the chromosomes pairs, Ag-NORs chromosomes are presented in the box.

cies, and variations of this character were considered derived. In the present study all species analyzed presented single Ag-NORs, suggesting the maintenance of this basal condition. The size polymorphisms observed in most species analyzed, suggests that these polymorphisms occurred independently of the species systematic position.

Acknowledgments

The authors are grateful to Renato Devidé for technical assistance. This research was supported by the Brazilian agencies FAPESP (Fundação de Apoio à Pesquisa do Estado de São Paulo) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

References

Alves AL (2005) Análise da Evolução da Família Loricariidae (Telesotei, Siluriformes) Com Base em Caracteres Cromossômicos e de Seqüências de DNA. PhD Thesis, Botucatu (SP), Universidade Estadual Paulista.

Alves AL, Oliveira C, Foresti F (2003) Karyotype variability in eight species of the subfamilies Loricariinae and Ancistrinae (Teleostei, Siluriformes, Loricariidae). Caryologia 56: 57–63.

Alves AL, Oliveira C, Foresti F (2005) Comparative cytogenetics analyses in eleven species of the subfamilies Neoplecostominae and Hypostominae (Siluriformes, Loricariidae). Genetica 124: 127–136. doi: 10.1007/s10709-004-7561-4

Alves AL, Oliveira C, Nirchio M, Granado A, Foresti F (2006) Karyotypic relationships among the tribes of Hypostominae (Siluriformes: Loricariidae) with description of XO sex chromosome system in a Neotropical fish species. Genetica 128: 1–9. doi: 10.1007/s10709-005-0715-1

Armbruster JW (2004) Phylogenetic relationships of the suckermouth armoured catfishes (Loricariidae) with emphasis on the Hypostominae and the Ancistrinae. Zoological Jounal Linnean Society 141: 1–80. doi: 10.1111/j.1096-3642.2004.00109.x

Artoni RF, Bertollo LAC (1996) Cytogenetic studies on Hypostominae (Pisces, Siluriformes, Loricariidae). Considerations on karyotype evolution in the genus *Hypostomus*. Caryologia 49(1): 81–90. doi: 10.1023/A:1003957719178

Artoni RF, Betollo LAC (1999) Nature and distribution of constitutive heterochromatin in fishes, genus *Hypostomus* (Loricariidae). Genetica 106: 209–214.

Bitencourt JA, Affonso PRAM, Giuliano-Caetano L, Dias AL (2011) Identification of distinct evolutionary units in allopatric populations of *Hypostomus* cf. *wuchereri* Günther, 1864 (Siluriformes: Loricariidae): karyotypic evidence. Neotropical Ichthyology 9(2): 317–324. doi: 10.1590/S1679-62252011000200008

Fontana F, Lanfredi M, Congiu L, Leis M, Chicca M, Rossi R (2003) Chromosomal mapping of 18S-28S and 5S rRNA genes by two-colour fluoirescent in situ hybridization in six sturgeon species. Genome 46: 473–477. doi: 10.1139/g03-007

Foresti F, Almeida-Toledo LF, Toledo-Filho SA (1981) Polymorphic nature of nucleolus organizer regions in fishes. Cytogenetic and Cell Genetic 31: 137–144. doi: 10.1159/000131639

Foresti F, Oliveira C, Almeida-Toledo LF (1993) A method for chromosome preparations from large specimens of fishes using in vitro short treatment with colchicine. Experientia 49: 810–813. doi: 10.1007/BF01923555

Giuliano-Caetano L (1998) Polimorfismo cromossômico Robertsoniano em populações de *Rineloricaria latirostris* (Pisces, Loricariinae). PhD Thesis, São Carlos (SP), Departamento de Ciências Biológicas, Universidade Federal de São Carlos. 78 p.

Kavalco KF, Pazza R, Bertollo LAC, Moreira-Filho O (2004) Heterochromatin characterization of four species of the family Loricariidae (Siluriformes). Hereditas 141: 1–6.

Kavalco KF, Pazza R, Bertollo LAC, Moreira-Filho O (2005) Karyotypic diversity and evolution of Loricariidae (Pisces, Siluriformes). Heredity 94: 180–186. doi: 10.1038/sj.hdy.6800595

Mendes-Neto EO, Vicari MR, Artoni RF, Moreira-Filho, O (2011) Description of karyotype in *Hypostomus regani* (Ihering, 1905) (Teleostei, Loricariidae) from the Piumhi river in Bra-

zil with comments on karyotype variation found in *Hypostomus*. Comparative Cytogenetic 5(2): 133–142. doi: 10.3897/compcytogen.v5i2.964

Oliveira C, Gosztonyi AE (2000) A cytogenetic study of *Diplomystes mesembrinus* (Teleostei, Siluriformes, Diplomystidae) with a discussion of chromosome evolution in siluriformes. Caryologia 53(1): 31–37.

Paintner-Marques TR, Giuliano-Caetano L, Dias AL (2002) Multiple NORs in *Bryconamericus* aff. *exodon* (Osteichthyes, Characidae, Tetragonopterinae). Hereditas 137: 107–112. doi: 10.1034/j.1601-5223.2002.01651.x

Porto-Foresti F, Oliveira C, Tabata YA, Rigolino MG, Foresti F (2002) NORs inheritance analysis in crossing including indivisuals from two stocks of rainbow trout (*Oncorhynchus mykiss*). Hereditas 136: 227–230. doi: 10.1034/j.1601-5223.2002.1360308.x

Reis RE, Kullander SO, Ferraris Jr CJ (2003) Check list of the freshwater fishes of South America. Porto Alegre: Edipucrs, 729 pp.

Rubert M, Zawadzki CH, Giuliano-Caetano L (2008) Cytogenetic characterization of *Hypostomus nigromaculatus* (Siluriformes: Loricariidae). Neotropical Ichthyology 6(1): 93–100. doi: 10.1590/S1679-62252008000100011

Wasko AP, Galetti Jr M (2000) Mapping 18S ribosomal genes in fish of the genus *Brycon* (Characidae) by fluorescence in situ hybridization (FISH). Genetic and Molecular Biology 23(1): 135–138. doi: 10.1590/S1415-47572000000100025

CompCytogen 6(3): 323–333 (2012)
doi: 10.3897/CompCytogen.v6i3.3637
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Cytogenetic analysis in *Thoracocharax stellatus* (Kner, 1858) (Characiformes, Gasteropelecidae) from Paraguay River Basin, Mato Grosso, Brazil

Edson Lourenço da Silva[1], Rafael Splendore de Borba[1], Liano Centofante[2], Carlos Suetoshi Miyazawa[3], Patrícia Pasquali Parise-Maltempi[1]

1 *Instituto de Biociências, UNESP Univ Estadual Paulista, Campus de Rio Claro, Departamento de Biologia, Laboratório de Citogenética. Av. 24A, 1515. CEP: 13506-900. Rio Claro, SP, Brazil* **2** *Instituto de Biociências, UFMT Univ Federal de Mato Grosso, Departamento de Biologia e Zoologia. Laboratório de Citogenética Animal. Av. Fernando Corrêa da Costa s/n, CCBS-II, CEP: 78060-900. Cuiabá, MT, Brazil* **3** *Universidade Federal do ABC, Centro de Ciências Naturais e Humanas (CCNH). Rua Santa Adélia, 166. Bairro Bangu, CEP 09.210-170. Santo André, SP, Brazil*

Corresponding author: *Patrícia Pasquali Parise-Maltempi* (parise@rc.unesp.br)

Academic editor: *G. Furgala-Selezniow* | Received 6 July 2012 | Accepted 11 September 2012 | Published 26 September 2012

Citation: Silva EL, Borba RS, Centofante L, Miyazawa CS, Parise-Maltempi PP (2012) Cytogenetic analysis in *Thoracocharax stellatus* (Kner, 1858) (Characiformes, Gasteropelecidae) from Paraguay River Basin, Mato Grosso, Brazil. Comparative Cytogenetics 6(3): 323–333. doi: 10.3897/CompCytogen.v6i3.3637

Abstract

Thoracocharax stellatus (Characiformes, Gasteropelecidae) is a small Neotropical species of fish, widely distributed in several rivers of South America. Evidence for karyotype heteromorphysm in populations from different geographical regions has been reported for this species. In this way, populations of *T. stellatus* from the Paraguay River basin were cytogenetically characterized and the results were compared with other studies performed in the same species but from different basins. The results showed a diploid number of 2n = 54 for *T. stellatus,* with chromosomes arranged in 6 metacentric (m), 6 submetacentric (sm), 2 subtelocentric (st) and 40 acrocentric (a), for both sexes, with a simple Nucleolus Organiser Region (NOR) system reported by the techniques of silver nitrate impregnation and fluorescent *in situ* hybridisation (FISH) using 18S rDNA sequences as probe. The distribution of constitutive heterochromatin, observed by the C-band technique and Chromomycin A3 staining showed great similarity among the analyzed populations and consists mainly of discrete blocks in the pericentromeric and telomeric regions of most chromosomes. The presence of female heterogamety was also observed indicating a ZZ/ZW system with W chromosome almost totally heterochromatic. The results also show cytogenetic diversity of the group and are useful to understand the mechanisms of karyotype evolution of the family.

Keywords
Thoracocharax stellatus, Ag-NOR, C-band, FISH, 18S rDNA

Introduction

The family Gasteropelecidae (Characiformes) comprises a group of small Neotropical fishes that inhabit rivers of the main Central and South America basins, with exception of the southeast of Brazil and Chile (Géry 1977, Weitzman and Palmer 2003). Three genera are recognized in this family (*Carnegiella* Eigenmann, 1909, *Gasteropelecus* Scopoli, 1777 and *Thoracocharax* Fowler, 1906), comprising a total of nine nominal species.

The first taxonomic study regarding this family was conducted by Weitzman (1954, 1960) which suggested, by osteological observation, the genus *Thoracocharax* represents an independent lineage inside the group, named as tribe Thoracocharacini. The genera *Gasteropelecus* and *Carnegiella* comprise a sister group belonging to a second lineage named tribe Gasteropelecini (Weitzman 1960).

Despite the reduced number of species, this family presents many taxonomic problems related to the difficulty of finding species-specific characters. *Carnegiella marthae* (Myers, 1927) e.g. have osteological differences related to anal fin rays among specimens from Orinoco and Negro rivers, and the specimens from Peruvian Amazon and Madeira Rivers (Géry 1977). So the typical form from Orinoco-Negro has been called *Carnegiella marthae marthae*, and the species from Peruvian Amazon called *Carnegiella marthae schereri* (Géry 1977).

Thus, considering its large distribution, an accurate analysis of specimens from different localities can reveal the existence of putative new species (Weitzman and Weitzman 1982, Weitzman and Palmer 1996, Weitzman and Palmer 2003).

As regards to the cytogenetic aspects, the family Gasteropelecidae is relatively non-conserved, with diploid numbers varying from 2n=48 to 2n=54. The species studied until now are recognized by the presence of many subtelocentric and acrocentric chromosomes and almost all presenting one chromosome pair carrier the NOR, that can be variable only in some populations (Carvalho et al. 2002). Also been described and characterized in the family the sexual system ZZ/ZW for the genera *Carnegiella* and *Thoracocharax*.

The genus *Thoracocharax* is the most widely distributed among the gasteropelecids, and is characterized by species that have distinct chromosomal and morphological characteristics in different geographic regions. *Thoracocharax stellatus* (Kner, 1858), for example, presents a considerable morphological polymorphism among populations, mainly due to the geographic isolation (Silva et al. 2009)

The karyotypic diversity observed in *T. stellatus* comprises different diploid numbers and polymorphism in NOR (Nucleolus Organiser Region) number and different chromosomes involved in the sexual differentiation (ZZ/ZW system) (Carvalho et al. 2002, Venere et al. 2007). These variations can help determining the taxonomic state of some populations.

Due to the cytogenetic diversity already observed for different *T. stellatus* populations, the objective of this study was to describe the karyotypic structure of three populations from the Paraguay basin not studied yet and identify chromosomal markers for this species by establishing chromosomal variation patterns among different populations.

Material and methods

For the cytogenetic analysis of *T. stellatus,* 111 specimens collected in the Paraguay basin were used, 38 from Cuiabá River - São Gonçalo (SG) (15°39'9.96"S, 56°4'8.62"W), 26 in Cuiabá River - SESC (SE) (16°38'55.0"S, 56°28'06.2"W) and 47 from Barra do Bugres (BB) (15°4'41.13"S, 57°10'55.64"W) (Fig. 1). The material collected was deposited in the Laboratório de Citogenética Animal/UFMT, Mato Grosso (LCA 018, LCA 023, LCA 028).

Direct cytological preparations were produced from kidney samples according to the methods given by Foresti et al. (1981). Karyotype analysis was conducted after conventional staining with Giemsa.

The chromosomes were morphologically classified according to the nomenclature proposed by Levan et al. (1964), with the following modifications: metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a). Heterochromatic regions were visualized by C-banding (Sumner 1972) and double fluorochrome staining (Chromomycin A3 + 4,6 – diamide 2 phenylindole - DAPI), according to the methods used by Schweizer (1980) and NORs were detected by silver nitrate impregnation (Ag-NORs), according to the procedure described by Howell and Black (1980).

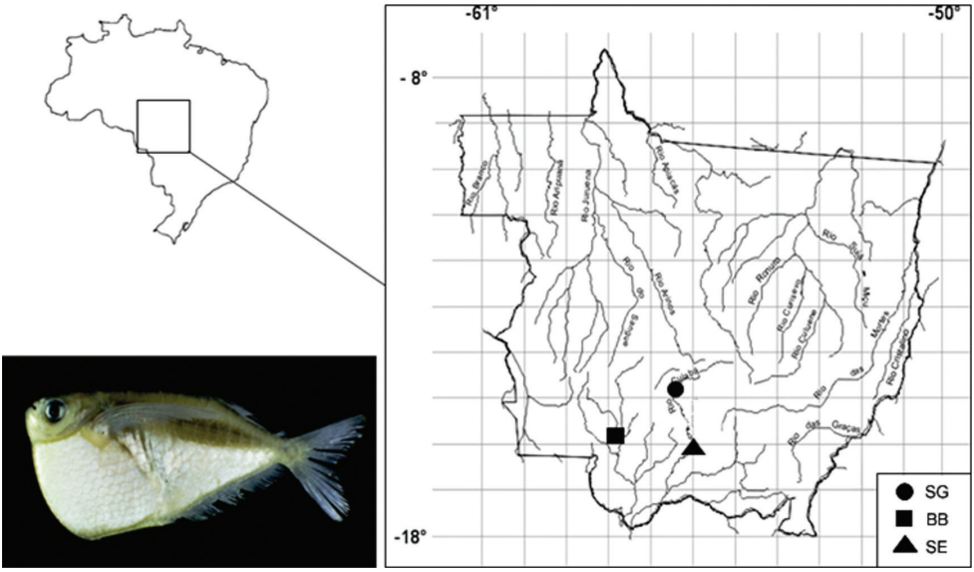


Figure 1. Sampling sites of the three populations of *T. stellatus*. SG: São Gonçalo; SE: SESC and BB Barra do Bugres.

The Fluorescent *in situ* hibridization (FISH) analyses were performed basically according to the method of Pinkel et al. (1986) with some modifications according to Silva et al. (2012). The 18S rDNA probe was obtained by PCR using specific primers set (NS1 5'-GTAGTCATATGCTTGTCTC-3' and NS2 5'-GGCTGCTGGCAC-CAGACTTGC-3') and labelled with digoxigenin by PCR. Metaphase chromosome slides were incubated with RNAse (40 µg/ml) for 1 h at 37°C and dehydrated using an ethanol series (70%, 85%, 100%). Chromosomal DNA was denatured for 1 min and 45 seconds in 70% formamide/2× SSC at 70°C and the spreads were dehydrated using the same ethanol series. The hybridisation solution (50% formamide/2× SSC, 10% dextran sulfate, and 1.5 µg/ml DNA probe) was denatured for 10 min at 95°C and applied to each slide under a coverslip. The hybridisation was performed overnight at 37°C in a moist chamber containing 2× SSC. Post-hybridisation washes were conducted using: 50% formamide/2× SSC (pH 7.0) twice for 5 min at 45°C; 2× SSC at 45°C; and 2× SSC at room temperature. Signals were detected using antidigoxigenin-rhodamine antibody. Chromosomes were counterstained with DAPI (1.5 µg/mL) and mounted in antifading solution.

Chromosomes were observed using an Olympus BX51 microscope coupled to an Olympus digital camera model D71. Chromosome images were captured using the DP Controller software.

Results

Table 1 summarizes the karyotypic data obtained in the present study as well as in the available literature. The *T. stellatus* specimens from three localities of the Paraguay River basin present diploid number 2n=54 chromosomes, with fundamental number NF=68 and karyotypic formulae that includes 6m, 6sm, 2st and 40a for both sexes (Figure 2 A, B).

The distribution pattern of constitutive heterochromatin presents similarity for all populations and is composed by discrete blocks mainly at the telomeric and pericentromeric regions in the majority of the chromosomes (Figure 2 C, D). The only

Table 1. Chromosomal data on Gasteropelecidae from different Brazilian hydrographic basins.

					karyotypic formula				
Species	**Hydrographical basin**	**Sex chromosomes**	**Ag-NOR**	**2n**	**m**	**sm**	**st**	**a**	**Ref**
Thoracocharax stellatus	Araguaia	ZZ/ZW	2–4	54	6	6	6	36	1
T. stellatus	Paraguay	ZZ/ZW	2	54	6	6	2	40	2
T. cf. *stellatus*	Amazon	ZZ/ZW	2 ▲	52	8	16	4	24	3
Carnegiella marthae	Amazon	ZZ/ZW	2	50	20	12	4	14	4
C. strigata	Amazon	-	2–4	50	4	4	2	40	4

▲= Presence of size heteromorphisms. References= 1-Venere et al. (2007), 2- present paper, 3- Carvalho et al. (2002), 4- Terêncio et al. (2008)

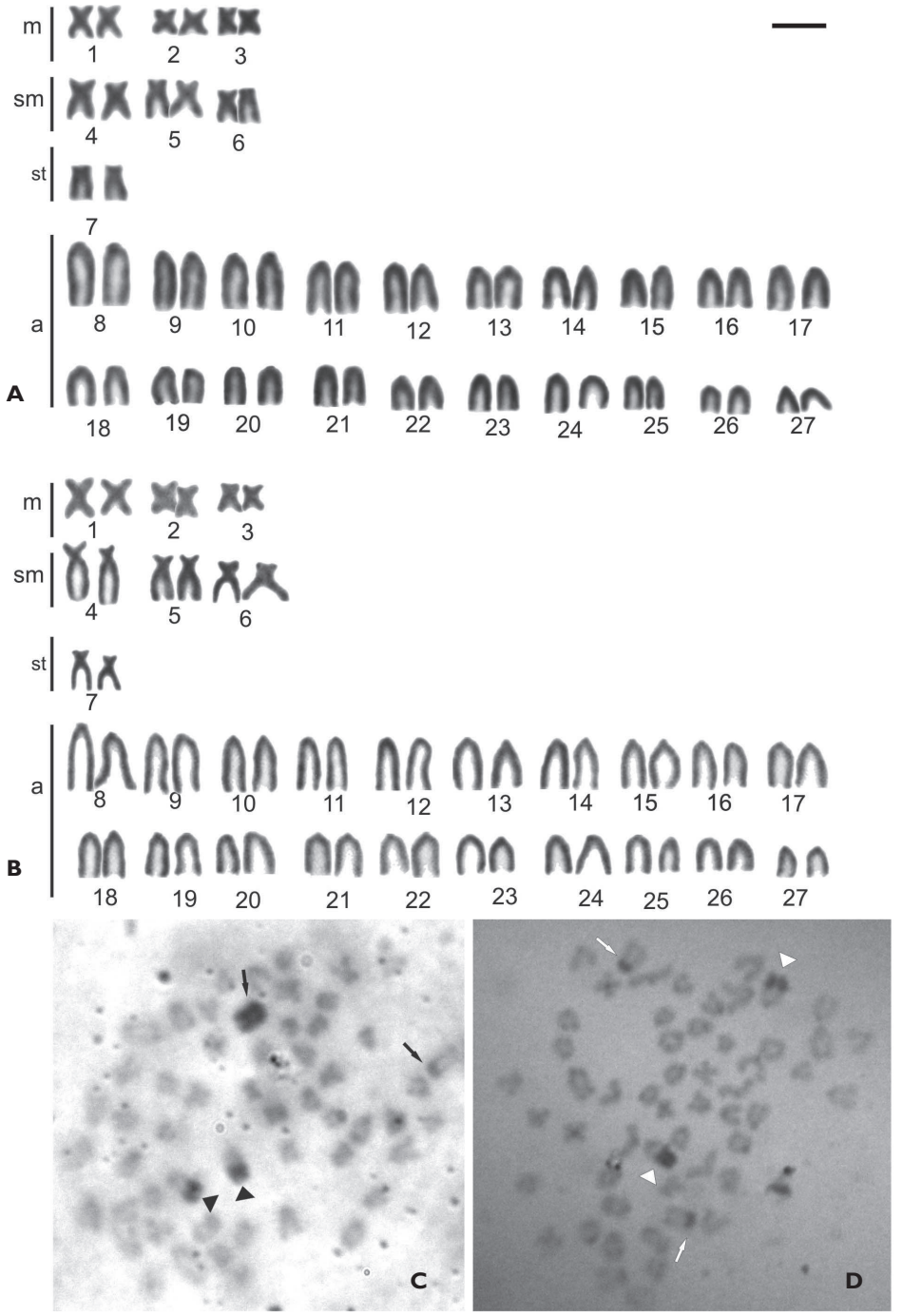


Figure 2. Female (**A**) and male (**B**) karyotype of *T. stellatus* stained with Giemsa showing 2n=54 chromosomes. Metaphases from female (**C**) and male (**D**) of *T. stellatus* showing heterochromatic blocks after C-band treatment. Arrows indicate the sex chromosomes Z and W in females and males Z and Z; arrowheads indicate the NOR chromosome. Bar = 10 μm.

exception was one acrocentric chromosome totally heterochromatic present in female karyotype, while the other chromosome of the pair presents a remarkable marker only in the pericentromeric region.

The male individuals present the conspicuous heterochromatin blocks in the pericentromeric region in corresponding chromosomes, indicating the presence of ZZ/ZW chromosome sex system in the studied populations (Figure 2 C, D).

After colloidal silver nitrate treatment, was evidenced only one pair carrier the NOR. This structure is restricted to the terminal region of an acrocentric pair, confirmed by FISH technique (Figure 3 B, E, G). The heterochromatic segments of sex chromosomes, as well as NOR presents bright signals through Chromomycin A_3 treatment, indicating a GC rich regions (Figure 3 C, F).

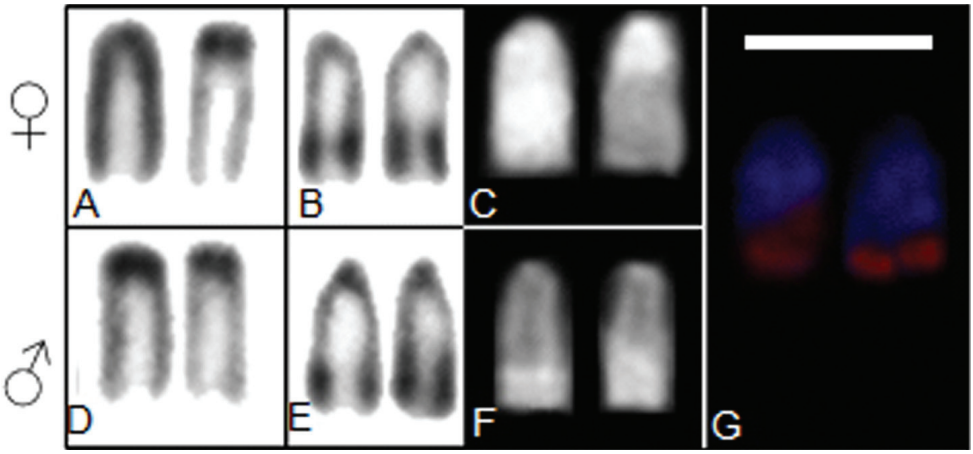


Figure 3. Chromosome markers from males and females individuals of *T. stellatus* **A** C-band of Z and W chromosomes of female **B** Ag-NOR bearing chromosomes of female individuals **C** CMA_3 treatment of Z and W chromosomes of female **D** C-band of ZZ chromosomes of male **E** Ag-NOR bearing chromosomes of male individuals **F** CMA_3 treatment of ZZ chromosomes of male **G** FISH 18S rDNA. Bar = 10 μm.

Discussion

In contrast to the situation observed in many families of the order Characiformes, the presence of non conserved karyotype is a common characteristic of Gasteropelecidae species. The diploid numbers range from 2n=48 to 2n=54 chromosomes (Hinegardner and Rosen 1972, Scheel 1973, Terêncio et al. 2008), being mostly, subtelocentric and acrocentric chromosomes (Carvalho et al. 2002).

The *T. stellatus* populations analyzed in the present study presented 2n=54 chromosomes in both sexes, similar to the observed for individuals from Araguaia- Tocantins basin (Venere et al. 2007). However, differences related to karyotypic formulae among these populations were observed, as diploid number, frequency of subtelocentric and acrocentric pairs and the presence of multiple NOR system (see Table 1). According to Centofante et al. (2003), cytogenetic studies in fishes have show that populations iso-

lated by geographic barriers are more favourable to the establishment of chromosome alterations. Thus, mechanisms of distance isolation together with historic events have been the principal factors influencing the changes among populations from Amazon and Araguaia-Tocantins basin (Venere et al. 2007), which can also explain the differentiation process observed among the populations from Araguaia and Paraguay rivers.

The NORs have been frequently employed as an important chromosome marker in many fish groups and their changes have been used as an important tool for the identification of chromosome markers among certain species and populations. The *T. stellatus* populations studied presented a single NOR system, but a number and size heteromorphysm of these structures was detected in other populations already studied (Table 1). Considering that the karyotypic composition of the Paraguay and Araguaia basins is stable within each population, the variation in number of active sites in these populations can be explained by a possible differential expression of rDNA cistrons as observed in studies of other group of fishes in other basins (Vicari et al. 2006).

According the results with base-specific fluorochromes the heterochromatin observed in *T. stellatus* is distributed in blocks containing different proportions of the nucleotides. The first kind, present in few chromosomes of complement, is rich in GC bases. In this group, the heteromorphic chromosomes related to the ZZ/ZW sex system and the NOR chromosomes are included. The W chromosome has a large, CMA_3+ positive heterochromatic fluorescent segment and in the Z only the pericentromeric regions is evidently heterochromatic. The sex chromosome system in *T. stellatus* from the Paraguay basin does not present explicit differences in size between Z and W chromosomes, unlike that observed in populations from other hydrographic basins.

The second kind of heterochromatin, evidenced through C-band technique is characterized by markers located in pericentromeric and telomeric regions of several chromosomes of the complement, without fluorescent signals after the colouring with Chromomycin A_3. This distribution pattern of heterochromatic blocks was already observed in several studies of chromosome characterization of many groups of fishes, as *Leporinus* (Margarido and Galetti 2000), *Astyanax* (Vicari et al. 2008), *Imparfinis* (Margarido and Moreira-Filho 2008) and *Hypostomus* (Rubert et al. 2008).

The female heterogamety is the most frequently observed sex determination system observed in fishes (Centofante et al. 2002). Depending on the group, the differentiation between Z and W chromosomes can be made by several ways: In some species there is an accumulation of heterochromatic segments followed by an increase of W chromosome in relation to Z chromosome (Centofante et al. 2001), accumulation of heterochromatin followed by loss of DNA sequences (Bertollo and Cavallaro 1992, Carvalho et al. 2002). In other species, substitutions of heterochromatic segments (Centofante et al. 2002) and rearrangements followed or not by loss of heterochromatic material (Artoni et al. 1998, Almeida-Toledo et al. 2002, Rosa et al. 2006). The occurrence of a W chromosome totally heterochromatic in the *Thoracochara*x species studied until now suggests that its origin is related to the accumulation of heterochromatin in these chromosomes.

Phylogenetic relationships based on morphological characters among gasteropelecids and other families have been discussed since Eigenmann (1910) using morphological characters suggested that they are a sister group of Triportheinae and Cynodontidae. The morphological evidence of a possible relationship between Gasteropelecidae and Triportheinae could be enhanced by the description of the similar sex chromosomes in both groups (Artoni and Bertollo 2002, Carvalho et al. 2002, Nirchio et al. 2007, Venere et al. 2007, this study). Some authors suggest that this system is a characteristic established before the speciation of the group *Triportheus* (Artoni et al. 2001, Artoni and Bertollo 2002), so it is acceptable to admit that a similar event may have occurred in the family Gasteropelecidae.

The increase of new cytogenetic studies have shown that other species of Gasteropelecidae such as *Carnegiella marthae*, have 50 chromosomes, NOR polymorphisms and a ZZ / ZW sex differentiation system (Terêncio et al. 2008). In this group, however, the Z and W chromosomes have different size. This variation illustrates the importance of chromosome studies of other species of the family, not cytogenetically characterized yet. The molecular data available to date also reinforce the proximity of *Carnegiella* and *Thoracocharax* genus, according to Abe (2007) based in mtDNA sequence, these two genera together comprises a sister clade of the independent clade composed only of *Gasteropelecus* species.

Isolated events of chromosome rearrangements, without phylogenetic implications, as fission may be responsible to reduction of subtelocentric and increase of larges acrocentric chromosomes, resulting in the chromosomal particularities observed in *T. stellatus* from Paraguay Rivers basin in relation to other population already studied. The results also show the cytogenetic diversity of the group, is useful to understand the mechanisms of karyotype evolution of the family as well as understanding the processes of differentiation and evolution of sex chromosomes in the group.

Acknowledgments

The authors would like to thank CAPES, FAPEMAT, BASA and UFMT, for the financial support.

References

Abe KT (2007) Filogenia molecular de Gasteropelecidae (Ostariophysi: Characiformes) e estudo de suas relações com outros Characiformes. Phd Dissertation, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil. www.dominiopublico.gov.br/pesquisa/DetalheObraForm.do?select_action=&co_obra=43136

Almeida-Toledo LF, Daniel-Silva MFZ, Moisés CB, Fonteles SBA, Lopes CE, Akama A, Foresti F (2002) Chromosome evolution in fish: sex chromosome variability in *Eigenmannia virescens* (Gymnotiformes. Sternopygidae). Cytogenetic and Genome Research 99: 164–169. doi: 10.1159/000071589

Artoni RF, Venere PC, Bertollo LAC (1998) A heteromorphic ZZ/ZW sex chromosome system in fish, genus *Hypostomus* (Loricariidae). Cytologia 63: 421–425. doi: 10.1508/cytologia.63.421

Artoni RF, Falcão JN, Moreira-Filho O, Bertollo LAC (2001) An uncommon condition for a sex chromosome system in Characidae fish: distribution and differentiation of ZZ/ZW system in *Triportheus*. Chromosome Research 9: 449–456. doi: 10.1023/A:1011620226348

Artoni RF, Bertollo LAC (2002) Evolutionary aspects of the ZZ/ZW sex chromosome system in the Characidae fish, genus *Triportheus*: a monophyletic state and NOR location on the W chromosome. Heredity 89:15–19.

Bertollo LAC, Cavallaro ZI (1992) A highly differentiated ZZ/ZW sex chromosome system in a Characidae fish, *Triportheus guentheri*. Cytogenetic and Cell Genetic 60: 60–63. doi: 10.1159/000133296

Carvalho ML, Oliveira C, Foresti F (2002) Description of a ZZ/ZW sex chromosome system in *Thoracocharax* cf. *stellatus* (Teleostei, Characiformes, Gasteropelecidae). Genetic and Molecular Biology 25: 299–303. doi: 10.1590/S1415-47572002000300008

Centofante L, Bertollo LAC, Moreira-Filho O (2001) Comparative cytogenetics among sympatric species of Characidium (Pisces, Characiformes): diversity analysis with the description of ZW sex chromosome system and natural triploidy. Caryologia 54: 253–260.

Centofante L, Bertollo LAC, Moreira-Filho O (2002) A ZZ/ZW sex chromosome system in a new species of the genus *Parodon* (Pisces, Parodontidae). Caryologia 55: 139–150.

Centofante L, Bertollo LAC, Miyazawa CS, Moreira-Filho O (2003) Chromosomal differentiation in allopatric population of *Hyphessobrycon anisitsi* (Pisces, Tetragonopterinae). Cytologia 68: 283–288. doi: 10.1508/cytologia.68.283

Eigenmann CH (1910) Catalogue of the freshwater fishes of tropical and south temperate America. III Zoology. Reports of the Princeton University Expeditions to Patagonia, 1896 –1899. Princeton, pp. 375–511.

Foresti F, Toledo LF, Toledo SA (1981) Polymorfic nature of nucleolus organizer regions in fishes. Cytogenetic and Cell Genetic, 31: 137–144. doi: 10.1159/000131639

Géry J (1977) Characoids of the World. TFH publication, 672p.

Hinegardner R, Rosen DE (1972) Cellular DNA content and evolution of teleostean fishes. The American Naturalist 106: 621–644. doi: 10.1086/282801

Howell WM, Black DA (1980) Controlled silver staining nucleolus organizer regions with protective colloidal developer: a 1-step method. Experientia 36: 1014–1015. doi: 10.1007/BF01953855

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52: 201–220. doi: 10.1111/j.1601-5223.1964.tb01953.x

Margarido VP, Galetti PM (2000) Amplification of a GC-rich heterochromatin in the freshwater fish *Leporinus desmotes* (Characiformes, Anostomidae). Genetic and Molecular Biology 23: 569–573. doi: 10.1590/S1415-47572000000300012

Margarido VP, Moreira-Filho O (2008) Karyotypic differentiation through chromosome fusion and number reduction in *Imparfinis hollandi* (Ostariophysi, Heptapteridae). Genetic and Molecular Biology 31: 235–238. doi: 10.1590/S1415-47572008000200012

Nirchio M, Oliveira C, Ferreira IA, Granado A, Ron E (2007) Extensive polymorphism and chromosomal characteristics of ribosomal DNA in the characid fish *Triportheus venezuelen-*

sis (Characiformes, Characidae). Genetic and Molecular Biology 30: 25–30. doi: 10.1590/S1415-47572007000100007

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proceedings of the National Academy of Sciences USA 83: 2934–2938. doi: 10.1073/pnas.83.9.2934

Rosa R, Bellafronte E, Moreira-Filho O, Margarido VP (2006) Constitutive heterochromatin, 5S and 18S rDNA genes in *Apareiodon* sp. (Characiformes, Parodontidae) with a ZZ/ZW sex chromosome system. Genetica 128: 159–166. doi: 10.1007/s10709-005-5700-1

Rubert M, Zawadzki CH, Giuliano-Caetano (2008) Cytogenetic characterization of *Hypostomus nigromaculatus* (Siluriformes: loricariidae). Neotropical Ichthyology 6: 93–100. doi: 10.1590/S1679-62252008000100011

Scheel JJ (1973) Fish chromosome and their evolution. Internal Report of Denmarks Akvarium, Charlottenlund, Denmark 22.

Schweizer D (1980) Simultaneous fluorescent staining of R bands and specific heterochromatic regions (DA/DAPI bands) in human chromosomes. Cytogenetic and Cell Genetic 27:190–193. doi: 10.1159/000131482

Silva EL, Centofante L, Miyazawa CS (2009) Morphometrics analysis in *Thoracocharax stellatus* (Kner, 1858) (Characiformes, Gasteropelecidae) from different South American river basins. Biota Neotropica 9: 71–76. doi: 10.1590/S1676-06032009000200006

Silva EL, Piscor D, Ponzetto J, Centofante L, ALves AL, Parise-Maltempi PP (2012) Cytogenetic characterisation of the ornamental freshwater fish, *Piabucus melanostomus* (Iguanodectinae) from Brazilian wetlands and its relation with species of Characidae basal group. Reviews in Fish Biology and Fishers 22: 477–484. doi: 10.1007/s11160-011-9238-2

Sumner AT (1972) A simple technique for demonstrating centromeric heterocromatin. Experimental Cell Research 75: 304–306. doi: 10.1016/0014-4827(72)90558-7

Terencio ML, Schneider CH, Gross MC, Silva AM, Feldberg E, Porto JR (2008) Comparative cytogenetics of *Carnegiella marthae* and *Carnegiella strigata* (Characiformes, Gasteropelecidae) and description of a ZZ/ZW sex chromosome system. Genetic and Molecular Biology 31: 231–234. doi: 10.1590/S1415-47572008000200011

Venere PC, Souza IL, Martins C, Oliveira C (2007) Occurrence of ZZ/ZW sex chromosomes in *Thoracocharax stellatus* fish (Characiformes, Gasteropelecidae) from the Araguaia River, South America. Genetica 133: 109–112. doi: 10.1007/s10709-007-9194-x

Vicari MR, Pazza R, Artoni RF, Margarido VP, Bertollo LAC (2006) Cytogenetics and biogeography: considerations about the natural origin of *Hoplias malabricus* on the Iguaçu River. Brazilian Archives of Biology and Technology 49: 297–303. doi: 10.1590/S1516-89132006000300015

Vicari MR, Noleto RB, Artoni RF, Moreira-Filho O, Bertollo LAC (2008) Comparative cytogenetics among species of the *Astyanax scabripinnis* complex: Evolutionary and biogeographical inferences. Genetic and Molecular Biology 31: 173–179. doi: 10.1590/S1415-47572008000200002

Weitzman SH (1954) The oesteology and relationships of the South American characoid fishes of the subfamily Gasteropelecinae. Stanford Ichthyology Bulletin 4: 213–263.

Weitzman SH (1960) Further notes on the relationships of the South American characoid fishes of the subfamily Gasteropelecinae. Stanford Ichthyology Bull 7: 217–239.

Weitzman SH, Weitzman M (1982) Biogeography and evolutionary diversification. In: Prance GT (Ed) Neotropical freshwater fishes, with comments on the refuge theory. Columbia University Press, New York, 403–422.

Weitzman SH, Palmer L (1996) Do freshwater hatchetfishes really fly? Tropical Fish Hobbyist 45: 195–206.

Weitzman SH, Palmer L (2003) Family Gasteropelecidae. In: Reis et al. (Eds) Checklist of the Freshwater fishes of South and Central America. Porto Alegre, Edipucrs, 101–103.