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Chromosomal complements of some Atlantic Blennioidei and Gobioidei species (Perciformes)

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Abstract

A remarkable degree of chromosomal conservatism (2n=48, FN=48) has been identified in several families of Perciformes. However, some families exhibit greater karyotypic diversity, although there is still scant information on the Atlantic species. In addition to a review of karyotypic data available for representatives of the suborders Blennioidei and Gobioidei, we have performed chromosomal analyses on Atlantic species of the families Blenniidae, *Ophioblennius trinitatis* Miranda-Ribeiro, 1919 (2n=46; FN=64) and *Scartella cristata* (Linnaeus, 1758) (2n=48; FN=50), Labrisomidae, *Labrisomus nuchipinnis* (Quoy & Gaimard, 1824) (2n=48; FN=50) and Gobiidae, *Bathygobius soporator* (Valenciennes, 1837) (2n=48; FN=56). Besides variations in chromosome number and karyotype formulas, Ag-NOR sites, albeit unique, were located in different positions and/or chromosome pairs for the species analyzed. On the other hand, the heterochromatic pattern was more conservative, distributed predominantly in the centromeric/pericentromeric regions of the four species. Data already available for Gobiidae, Blenniidae and Labrisomidae show greater intra- and interspecific karyotypic diversification when compared to other groups of Perciformes, where higher uniformity is found for various chromosome characteristics. Evolutionary dynamism displayed by these two families is likely associated with population fractionation resulting from unique biological characteristics, such as lower mobility and/or specific environmental requirements.

Keywords

Chromosomal evolution, marine fish, Bleniidae, Gobiidae, Labrisomidae

Introduction

Although karyotypic characteristics for some families of marine fish are already known, information on groups of Perciformes is still significantly disproportionate. Among these, suborders Blennioidei and Gobioidei stand out because of the large number of species they represent.

Suborders Gobioidei, with 2,121 species, and Blennioidei with 732 species, are spread throughout the tropical zone, typically represented by small specimens with low mobility and the ability to withstand changes in temperature and salinity (Nelson 2006).

Species of Blennioidei and Gobioidei investigated (e.g. Cataudela et al. 1973; Garcia et al. 1987; Ene 2003) have shown sufficient chromosomal peculiarities for species discrimination and understanding of their evolutionary aspects. In some families, such as Blenniidae, Labrisomidae and Gobiidae, sharing cryptic morphological characteristics combined with poor knowledge of the biological characteristics for many species, contributes to the relative taxonomic inaccuracy of this group. As such, cytotaxonomic markers (Garcia et al. 1987; Caputo 1998; Caputo et al. 2001) and phylogenetic analyses based on molecular data (Wang et al. 2001; Thacker 2003; Gysels et al. 2004; Almada et al. 2005) have been increasingly used when assessing their kinship relations. Indeed, it has been suggested that phylogenetic analyses combine molecular and morphological data (Thacker 2003), as well as cytogenetic information. However, in light of the diversity in these groups, solid chromosome data are not yet sufficiently available, with only 7.5% of Bleniidae species and 4.5% of Gobioidei was karyotyped (Table 1). Despite the scarcity of data, a high degree of chromosomal polymorphism has been characterized among Gobiidae, primarily Robertsonian rearrangements (Caputo et al. 1999, Ene 2003), along with others such as tandem fusions and pericentric inversions (Giles et al. 1985; Thode et al. 1985; Amores et al. 1990).

The present study focuses on the karyotypic characterization of some Atlantic species of the families Blenniidae, *Ophioblennius trinitatis* Miranda-Ribeiro, 1919 and *Scartella cristata* (Linnaeus, 1758), Labrisomidae, *Labrisomus nuchipinnis* (Quoy & Gaimard, 1824) and Gobiidae, *Bathygobius soporator* (Valenciennes, 1837), through conventional chromosomal analysis, characterization of nucleolar organizer regions (Ag-NORs) and the distribution pattern of C-positive heterochromatin (C-banding) in chromosomes, discussing evolutionary aspects.

Material and methods

A total of 25 specimens of *Ophioblennius trinitatis* (7♂, 4♀ and 14 indeterminate), 11 specimens of *Scartella cristata* (4♂, 5♀ and 2 indeterminate), 13 specimens of *Labrisomus nuchipinnis* (4♂, 4♀ and 5 indeterminate) and 12 specimens of *Bathygobius soporator*, (5♂, 5♀ and 2 indeterminate) were used for chromosome analysis. *Ophioblennius trinitatis* specimens came from the coast of Rio Grande do Norte

Table 1. Cytogenetic data for Blennioidei and Gobioidei (Perciformes).

Suborder/ Family	Species	2n	Karyotype formula	FN	References
Blennioidei					
Blenniidae	*Aidablennius sphynx*	48	4m+4sm+40a	56	Cano et al. (1982)
	A. sphynx	48	2st+46a	50	Cataudella and Civitelli (1975)
	Atrosalarias fuscus	48	48a	48	Arai and Shiotsuki (1973)
	Blennius ocellaris	48	2m+2st+44a	52	Vitturi et al. (1986)
	B. ponticus	48	16sm+10st+22a	74	Garcia et al. (1987)
	B. yatabei	48	6sm+12st+30a	66	Arai and Shiotsuki (1974)
	Coryphoblennius galerita	48	2m+12sm+34a	62	Garcia et al. (1973)
	Dasson trossulus	40	8m+32st/a	48	Arai and Shiotsuki (1974)
	Istiblennius enoshimae	48	2m+46a	50	Arai and Shiotsuki (1973)
	I. lineatus	48	48st/a	48	Arai and Shiotsuki (1974)
	Lipophrys canevai	48	8st+40a	56	Cataudella and Civitelli (1975)
	L. pholis	46	8m+8sm+30a	62	Garcia et al. (1987)
	L. trigloides	46	4m+4sm+10st+28a	64	Cano et al. (1982)
	L. trigloides	48	2m+6sm+18st+22a	74	Cataudella and Civitelli (1975)
	L. trigloides	48	2m+22sm+2st+22a	74	Garcia et al. (1987)
	L. trigloides	48	2m+6sm+18st+22a	74	Vitturi et al. (1986)
	Omobranchus elegans	42	10m+2sm+6st+24a	60	Arai and Shiotsuki (1974)
	O. punctatus	44	4m+40a	48	Arai (1984)
	Ophioblennius trinitatis	46	6m+12st+28a	64	*Present study*
	Parablennius incognitus (=Blennius incognitus)	48	4st+44a	52	Cano et al. (1982)
	P. pilicornis (=Blennius pilicornis)	48	8st+40a	56	Catalano et al. (1985)
	P. gattorugine	48	2m+4sm+42a	54	Vitturi et al. (1986)
	P. pilicornis	48	48a	48	Brum et al. (1992)
	P. sanguinolentus	48	12st+36a	60	Cataudella et al. (1973)
	P. sanguinolentus	48	20sm+10st+18a	78	Garcia et al. (1987)
	P. tentacularis	48	48st/a	48	Vasil'ev (1985)
	P. tentacularis	48	1st+47a	49	Carbone et al. (1987)
	P. tentacularis	47	1sm+46a	48	Carbone et al. (1987)
	Salaria fluviatilis	48	48st/a	48	Cataudella and Civitelli (1975)
	S. pavo	48	8st+40a	56	Cataudella et al. (1973)
	S. pavo	48	16sm+14st+18a	78	Garcia et al. (1987)
	S. pavo	48	2st+46a	50	Vasil'ev (1980)
	Salarias faciatus	48	48a	48	Arai and Shiotsuki (1973)
	S. luctuosus	48	48st/a	48	Arai and Shiotsuki (1974)
	Scartella cristata (=Blennius cristatus)	48	2st+46a	50	Vitturi et al. (1986)
	S. cristata	48	2sm+46st/a	50	Brum et al. (1995)
	S. cristata	48	4st+44a	52	*Present study*
Gobioidei					
Clinidae	*Clinithracus argentatus*	48	2st+46a	50	Vitturi et al. (1986)
Labrisomidae	*Labrisomus nuchipinnis*	48	2sm+46a	50	Affonso (2000)
	L. nuchipinnis	48	2st+46a	50	*Present study*

Suborder/ Family	Species	2n	Karyotype formula	FN	References
Eleotridae	*Dormitator latifrons*	46	44m/sm+2st/a	90	Uribe-Alcocer et al. (1983)
	D. maculatus	46	34m/sm+12st/a	80	Maldonado-Monroy et al. (1985)
	D. maculatus	46	40m/sm+6st/a	86	Molina (2005)
	D. maculatus	46	14m+28sm+2st+2a(♀) 13m+28sm+3st+2a(♂)	90	Oliveira and Almeida-Toledo (2006)
	Eleotrioides strigatus	44	2m+42st/a	46	Arai and Sawada (1974)
	Eleotris acanthopomus	46	46st/a	46	Arai and Sawada (1974)
	E. picta	52	52a	52	Uribe-Alcocer and Diaz-James (1996)
	E. pisonis	46	2m/sm+42st/a	46	Uribe-Alcocer and Diaz-James (1996)
	E. pisonis	46	46a	46	Rocon-Stange (1992)
	E. pisonis	46	46a	46	Molina (2005)
	E. muralis	46	46a	46	Khuda-Bukhsh and Nayak (1990)
	Mogurnda mogurnda	46	6sm+40st/a	52	Arai et al. (1974)
	M. obscura	62	-	-	Nogusa (1960)
	Ophiocara porocephala	48	48a	48	Arai and Fujiki (1979)
	Oxyeleotris marmorata	46	2m+2sm+42a	50	Arai and Fujiki (1979)
Gobiidae	*Aboma latipes*	40	40a	40	Arai and Sawada (1974)
	Acanthogobius flavimanus	44	44st/a	44	Arai and Sawada (1974)
	A. flavimanus	44	36st+8a	80	Arai and Kobayashi (1973)
	A. flavimanus	44	10m/sm/st+34a	54	Arai and Sawada (1975)
	Acentrogobius pflaumi	50	48m/sm+2st/a	98	Nogusa (1960)
	Amblygobius albimaculatus	44	2m+42st/a	46	Nishikawa et al. (1974)
	Aphia minuta	44	44a	44	Caputo et al. (1999)
	A. minuta	43	42a+1st	42	Caputo et al. (1999)
	A. minuta	42	1m+1st+40a	44	Caputo et al. (1999)
	A. minuta	42	1M+1m+40a	44	Caputo et al. (1999)
	A. minuta	41	2M+1st+38a	44	Caputo et al. (1999)
	Apocryptes bato	46	24m+10sm+12a	80	Nayak and Khuda-Bukhsh (1987)
	A. lanceolatus	38	14m+22sm+2st	76	Nayak and Khuda-Bukhsh (1987)
	Awaous grammepomus	46	46st/a	46	Khuda-Bukhsh and Barat (1987)
	A. tajasica	46	46a	46	Stange and Passamani (1986)
	Bathygobius fuscus	48	48a	48	Arai and Sawada (1975)
	B. soporator	48	2m+46a	50	Brum et al. (1996)
	B. soporator	48	2m/sm+46a	50	Cipriano et al. (2002)
	B. soporator	48	2m+6st+40a	56	*Present study*
	B. stellatus	46	2st+44a	48	Vasil'ev (1985)
	B. stellatus	47	1sm+2st+43a	49	Vasil'ev (1985)
	Boleophthalmus boddaerty	46	46m/sm	92	Subrahmanyan (1969)
	B. glaucus	46	12m+20sm+2st+12a	80	Manna and Prasad (1974)
	B. pectinirostrus	46	46st/a	46	Arai and Sawada (1975)
	Bostrichthys sinensis	48	4m/sm+44a	52	Arai et al. (1974)
	Chaenogobius annularis	44	18sm+26st/a	62	Arai and Sawada (1975)
	C. annularis	44	36m/sm+8a	80	Arai et al. (1974)
	C. annularis	44	44a	44	Nogusa (1960)
	C. castaneus	44	36m/sm/st+8a	80	Nishikawa et al. (1974)
	C. isaza	44	12sm+32st/a	56	Arai and Sawada (1975)
	C. urotaenia	44	-	-	Nogusa (1960)

Suborder/ Family	Species	2n	Karyotype formula	FN	References
	C. urotaenia	42	14sm+28a	56	Yamada (1967)
	Chasmichthys dolichognatus	44	44st/a	44	Arai and Sawada (1975)
	C. gulosus	44	44st/a	44	Arai and Sawada (1975)
	C. gulosus	44	16m/sm/st+28a	60	Nishikawa et al. (1974)
	Ctenogobius criniger	50	34m/sm+6st+10a	90	Arai and Sawada (1974)
	Gillichthys mirabilis	44	12sm+32a	56	Chen and Ebeling (1971)
	G. seta	44	6m+14sm+24a	64	Chen and Ebeling (1971)
	Glossogobius fasciatopunctatus	44	10m+28sm+2st+4a	84	Fei and Tao (1987)
	G. giuris	46	46a	46	Rishi and Singh (1982)
	Gobiodon citrinus	44	2m+42st/a	46	Arai and Sawada (1974)
	G. citrinus	43	1m+42st/a	44	Arai and Sawada (1974)
	G.quinquestrigatus	44	44a	44	Arai and Fujiki (1979)
	G. rivulatus	44	44a	44	Arai and Fujiki (1979)
	Gobioides rubicundus	46	2m+26sm+10st+8a	84	Manna and Prasad (1974)
	Gobionellus shufeldti	48	48a (♀)	48	Pezold (1984)
	G. shufeldti	47	46a+1m (♂)	48	Pezold (1984)
	Gobiosoma macrodon	38	38a	38	Musammil (1974)
	G. zebrella	38	38a	38	Musammil (1974)
	Gobius abei	46	-	-	Nogusa (1960)
	G. bucchichi	44	2sm+42a	46	Thode and Alvarez (1983)
	G. cobitis	46	46a	46	Caputo et al. (1997)
	G. cruentatus	46	2st+44a	48	Thode and Alvarez (1983)
	G. fallax	38	8m/sm+30a	46	Thode et al. (1988)
	G. fallax	39	7m/sm+32a	46	Thode et al. (1988)
	G. fallax	40	6m/sm+34a	46	Thode et al. (1988)
	G. fallax	40	7m/sm+33a	47	Thode et al. (1988)
	G. fallax	41	5m/sm+36a	46	Thode et al. (1988)
	G. fallax	42	4m/sm+38a	46	Thode et al. (1988)
	G. fallax	43	3m/sm+40a	46	Thode et al. (1988)
	G. niger	52	2m+4sm+16st+30a	74	Vitturi and Catalano (1989)
	G. niger	51	3m+4sm+16st+28a	74	Caputo et al. (1997)
	G. niger	50	4m+4sm+16st+26a	74	Caputo et al. (1997)
	G. niger	49	5m+4sm+16st+24a	74	Caputo et al. (1997)
	G. paganellus	48	2sm+46a	50	Caputo et al. (1997)
	G. similis	44	?		Nogusa (1960)
	Gobiusculus flavescens	46	6m/sm+40a	52	Klinkhardt (1992)
	Luciogobius grandis	44	?		Arai (1981)
	L. guttatus	44	?		Arai and Kobayashi (1973)
	Mesogobius batrachocephalus	30	16m+14a	46	Ivanov (1975)
	Neogobius cephalarges	46	46a	46	Vasil'ev (1985)
	N. constructor	42	4m/sm+38a	46	Vasil'ev and Vasil'yeva (1994)
	N. cyrius	36	structural polymorphism		Vasil'ev and Vasil'yeva (1994)
	N. fluviatilis	46	46a	46	Vasil'ev (1985)
	N. eurycephalus	32	12m+2sm+18a	46	Ene (2003)

Suborder/ Family	Species	2n	Karyotype formula	FN	References
	N. eurycephalus	31	13m+2sm+16a	46	Ene (2003)
	N. eurycephalus	30	14m+2sm+14a	46	Ene (2003)
	N. gymnotrachelus	46	46a	46	Vasil'ev and Grigoryan (1992)
	N. kessleri	46	46a	46	Vasil'ev (1985)
	N. melanostomus	46	46a	46	Vasil'ev (1985)
	N. rhodionovi	46	46a	46	Vasil'ev and Vasil'yeva (1994)
	Odontamblyops rubicundus	46	4m+16sm+26st/a	66	Arai and Sawada (1975)
	Padogobius martensi	46	1m+3sm+2st+40a	52	Cataudella et al. (1973)
	Parioglossus raoi	46	46st/a	46	Webb (1986)
	Periophthalmus cantonensis	46	18m+12sm+16st/a	76	Arai and Sawada (1975)
	Pomatoschistus lozanoi	37	3m+12sm+10st+12a	62	Webb (1980)
	P. microps	46	4m+16sm+20st+6a	86	Klinkhardt (1989)
	P. minutus	46	4m+16sm+16st+10a	82	Klinkhardt (1989)
	P. minutus	46	18sm+18st+10a	82	Klinkhardt (1992)
	P. norvegicus	32	10m+10sm+8st+4a	60	Webb (1980)
	P. pictus	46	22m/sm+12st+12a	80	Klinkhardt (1992)
	Proterorhinus marmoratus	46	46a	46	Rab (1985)
	Pterogobius elapoides	44	14sm+30st	88	Arai and Kobayashi (1973)
	P. zonoleucus	44	14sm+30st	88	Arai and Sawada (1975)
	Quietula guaymasiae	42	6m+4sm+32a	52	Cook (1978)
	Q. y-cauda	42	42a	42	Cook (1978)
	Rhinogobius brunneus	44	44a	44	Nishikawa et al. (1974)
	R. flumineus	44	44a	44	Arai and Kobayashi (1973)
	R. giurinus	44	44a	44	Nishikawa et al. (1974)
	Rhodoniichthys laevis	42	16m/sm+26st	84	Arai et al. (1974)
	Sicyopterus japonicus	44	10m+10sm+24a	64	Arai and Fujiki (1979)
	Synechogobius hasta	44	2m+42st/a	46	Arai and Sawada (1975)
	Tridentiger obscurus	44	10m/sm+34a	54	Arai et al. (1974)
	T. trigonocephalus	44	28m/sm/st+16a	72	Arai et al. (1973)
	T. trigonocephalus	46	16sm+6st+24a	68	Fei and Tao (1987)
	Trypauchen vagina	46	12m+6sm+10st+18a	74	Khuda-Bukhsh (1978)
	Tukugobius flumineus	44	44a	44	Nadamitsu (1974)
	Zosterisessor ophiocephalus (= *Gobius ophiocephalus*)	46	46a	46	Vasil'ev (1985)
	Zosterisessor ophiocephalus (= *Gobius ophiocephalus*)	45	1st+45a	47	Vasil'ev (1985)
	Zosterisessor ophiocephalus	46	2m/sm+44a	48	Caputo et al. (1996)

(5°13'1.73"S; 35°9'57.85"W), northeastern Brazil (n=1), and the Saint Peter and Saint Paul (n=8) (00°55'02"N; 29°20'42"W) and Fernando de Noronha (n=16) (3°52'11"S; 32°26'13"W) archipelagos. The remaining specimens were collected on the coast of Rio Grande do Norte. Individuals were previously submitted to mitotic stimulation with compound attenuated antigens, for 24 to 48 hours (Molina 2001, Molina et al. 2010), anesthetized with clove oil (Eugenol) and sacrificed for the removal of anterior kidney fragments. Sexing of specimens was performed by macroscopic and microscopic examination of the gonads. Chromosome preparations were obtained from kidney

cells (Gold et al. 1990). Nucleolar organizer regions (NORs) were identified by stain with silver nitrate - Ag-NORs (Howell and Black 1980) and C-positive heterochromatin sites through C-banding (Sumner 1972).

Metaphase preparations were examined and photographed on an Olympus BX50 photomicroscope, using an Olympus DP70 digital camera system. Chromosomes were classified according to the position of the centromere in metacentrics (m), submetacentrics (sm), subtelocentrics (st) and acrocentrics (a) (Levan et al. 1964) and organized in order of decreasing size. The chromosome formula and FN (fundamental number or number of chromosomal arms) were established for each species, considering acrocentric chromosomes with a single arm and the remaining chromosomes exhibiting two arms.

Results

Cytogenetic analyses of Blenniidae species (Blennioidei)

Ophioblennius trinitatis showed 2n=46, with a chromosome formula equal to 6m+12st+28a (FN=64), irrespective of sex. Although chromosomes showed a gradual decline in size, the smallest acrocentric pairs corresponded to approximately one-third of the largest metacentric pairs. Nucleolar organizer regions are located in the terminal portions of the short arm on pair 9, the smallest subtelocentric pair. C-positive heterochromatin is discretely located in the centromeric/pericentromeric region of the chromosomes (Fig. 1a, b).

Scartella cristata showed 2n=48 chromosomes, with a chromosome formula equal to 4st+44a (FN=52). The karyotype also displays a gradual reduction in chromosome size. However, the largest chromosome pair exhibits only double the size in relation to the smallest karyotype pair. Ribosomal sites are located on the terminal portions of the short arms on chromosome pair 1. C-positive heterochromatin is also reduced and located in the centromeric regions of chromosomes (Fig. 1c, d).

Cytogenetic analyses of Labrisomidae and Gobiidae species (Gobioidei)

Labrisomus nuchipinnis (Labrisomidae) showed 2n=48 chromosomes with a chromosome formula of 2st+46a (FN=50), showing relatively more differentiated size between the largest and smallest chromosomes of the karyotype. Nucleolar organizer regions are in the terminal portions of the long arms on pair 2, corresponding to the largest pair of acrocentric chromosomes. C-positive heterochromatin was showed in the centromeric/pericentromeric region of all chromosome pairs, in relatively conspicuous blocks (Fig. 1e, f).

Bathygobius soporator (Gobiidae) also displayed the karyotype composed of 2n=48 chromosomes, but with the chromosome formula distinct from that of *L. nuchipinnis,* specifically, 2m+6st+40a (FN=56). Size difference between the largest and smallest

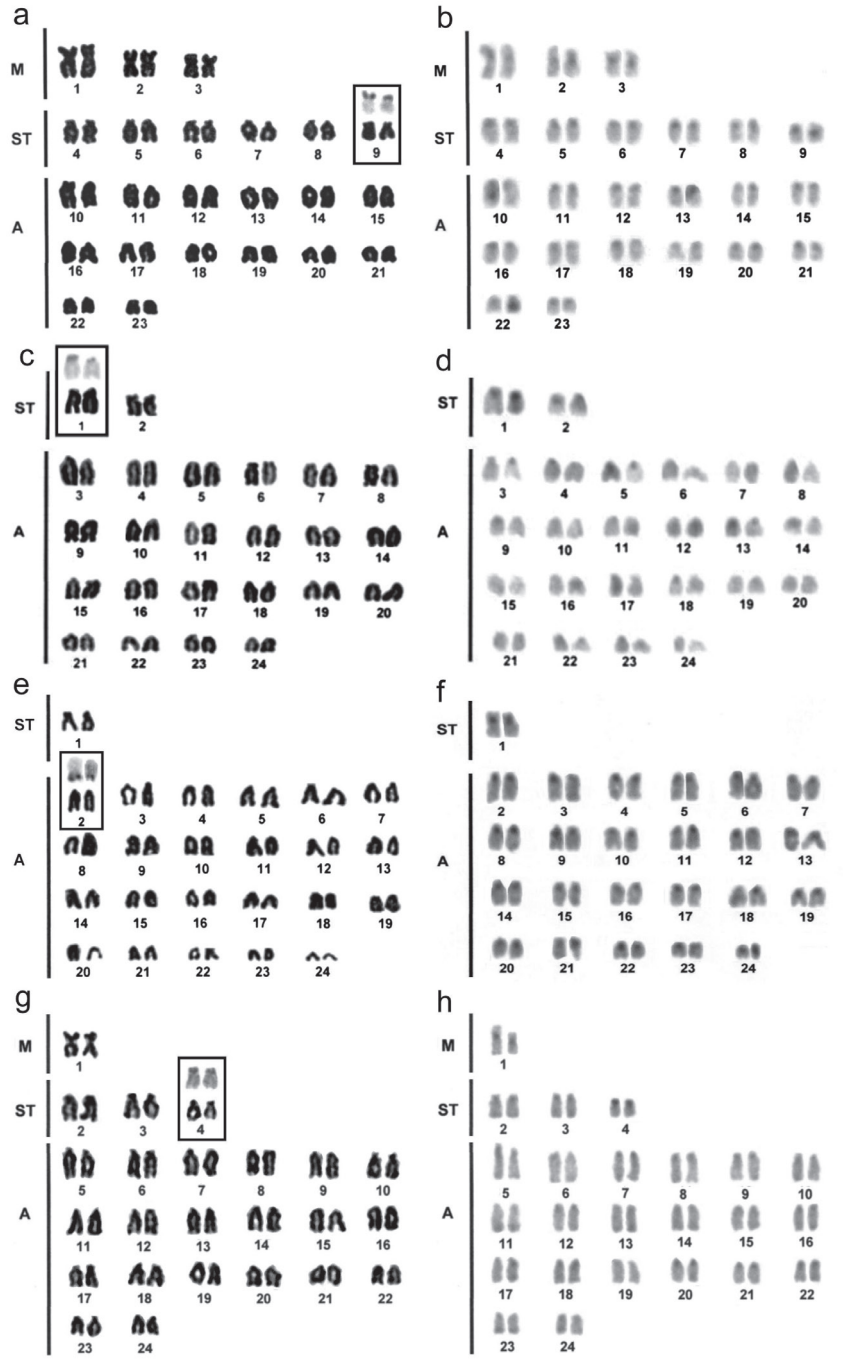

Figure 1. Karyotypes under Giemsa staining **a, c, e, g** and C-banding **b, d, f, h** of *Ophioblenius trinitatis;* **a, b** *Scartella cristata;* **c, d** *Labrisomus nuchipinnis;* **e, f** and *Bathygobius soporator;* **g, h** Ag-NOR-bearing chromosome pairs are highlighted.

chromosomes of the karyotype was far less pronounced. Ribosomal sites were on the terminal portions of the short arms on chromosome pair 4. C-banding showed discrete heterochromatic regions in the centromeric regions of most chromosomes and telomeric regions of some acrocentric pairs (Fig. 1g, h).

Discussion

Though many perciform families display a conserved karyotype pattern, with 2n=48 acrocentric chromosomes, some groups demonstrate dynamic tendencies in relation to chromosome evolution (Molina 2007). Much of identifiable chromosome diversity is attributed to pericentric inversions, the most common mechanism of chromosome evolution in this order (Galetti et al. 2000, 2006).

Representatives of the suborder Blennioidei (e.g., Carbone et al. 1987) and Gobioidei (e.g., Arai and Sawada 1974, 1975; Thode et al. 1988; Oliveira and Almeida-Toledo 2006) stand out for their greater karyotype variability and diversity. This includes species with conserved karyotyes and those that are highly diversified.

Within the Blennioidei, the Blenniidae, a monophyletic family, is divided into six tribes including Salariini and Parablenniini which, in turn, include the Atlantic species *O. trinitatis* and *S. cristata* respectively (Nelson 2006). Comparisons of mitochondrial DNA sequences in samples of *Ophioblennius* Gill, 1860 collected throughout the Atlantic suggest that the genus consists of six distinct lineages. One of these corresponds to species found in the Pacific, while the rest are recorded in the biogeographic provinces of the Atlantic: Brazilian, Caribbean, Mid-Atlantic, Sao Tome and Azores/Cape Verde (Muss et al. 2001). Chromosome characteristics reported here for *O. trinitatis* are the first for the genus, exhibiting 2n=46, 6m+12st+28a and FN=64. The relatively low diploid number and higher fundamental number in relation to the mean of other species of Blenniidae (Table 1), as well as the presence of large metacentric chromosomes, suggests pericentric inversion events and the occurrence of Robertsonian translocation involving two of its chromosome pairs. In turn, *S. cristata*, while also belonging to the family Blenniidae, has a distinct karyotype of 2n=48, 4st+44a and FN=52. Thus, *S. cristata* differs from *O. trinitatis* in that it contains an extra pair of chromosomes, lacks metacentric chromosomes and has different numbers of subtelocentric and acrocentric chromosomes in the karyotype. The karyotype of the *S. cristata* population studied here differs from the karyotypes previously described for the coastal population of Rio de Janeiro (SE Brazil), with 2sm+46st/a (Brum et al. 1994), and the Mediterranean population, with 2st+46a (Vitturi et al. 1986). Nevertheless, despite the growing number of discordant karyotype descriptions between populations on the NE and SE coasts of Brazil, one cannot rule out that these differences may arise from the difficulty in precisely defining types of cryptic chromosomes in the karyotype of this species.

In spite of displaying relative diversity in chromosome structure, only 18.5% of Blennioidei species exhibit differences in the basal diploid number, 2n=48 chromosomes. As shown in table 1, diploid numbers for representatives of this suborder vary

between 2n=40, found in *Dasson trossulus* (Jordan & Snyder, 1902) (Arai and Shiotsuki 1974) and 2n=52 in *Gobius niger* Linnaeus, 1758 (Vitturi and Catalano 1989), but with a conspicuous modal value of 2n=48.

In contrast to Blennioidei, suborder Gobioidei shows much more dynamic karyotype evolution, demonstrating highly variable karyotype patterns, where the diploid number ranges from 2n=30 for *Neogobius eurycephalus* (Kessler, 1874) (Ene 2003), to 2n=62 in *Mogurnda mogurnda* (Richardson, 1844) (Nogusa 1960). Cytogenetic data for 95 species show that only 9.6% have 2n=48 chromosomes, whereas the highest frequencies observed correspond to 2n=46 in 40% of species investigated, and 2n=44 in 32% (Table 1). As such, both Gobioidei species studied here are included in the group showing 2n=48 chromosomes, *L. nuchipinnis* with 2st+46a and FN=50 and *B. soporator* with 2m+6st+40a and FN=56. Thus, *B. soporator* differs from *L. nuchipinnis* in the presence of metacentric chromosomes and different numbers of subtelocentric and acrocentric chromosomes in the karyotype.

Among chromosome rearrangements involved in karyotypic differentiation of Gobiidae, Robertsonian fusions stand out, and are likely the most common event in this group (Amores et al. 1990; Galetti et al. 2000). However, other more complex changes in karyotypic structure (Thode et al. 1988; Vitturi and Catalano 1989; Caputo et al. 1997; Caputo et al. 1999), as well as the presence of different sex chromosomes (e.g., Pezold 1984; Baroiller et al. 1999), can also be observed, corroborating the high dynamic evolution that characterizes suborder Gobioidei. It has been suggested that the baseline/ancestral karyotype for Gobiidae would consist of 2n=46 acrocentric chromosomes (Vasil'ev and Grigoryan 1993), from which an increase in bi-brachial chromosomes would characterize more derived karyotypes. Based on this proposal, *B. soporator* (FN=56) would experience a greater number of structural rearrangements during its karyotypic evolution process in relation to *L. nuchipinnis* (FN=50).

Location and frequency of Ag-NOR sites are efficient cytotaxonomic markers in many groups of fish (Caputo 1998). Among species of Gobiidae, at least six different arrangement patterns for nucleolar organizer regions have been identified (Fig. 2), which supports the occurrence of intense karyotypic diversification mechanisms in this group. Thus, Ag-NOR sites can be found (a) in the telomeric region on the short arm of a single pair of acrocentric chromosomes, as in *Gobius fallax* Sarato, 1889 (Thode et al. 1983) and *Gobius paganellus* Linnaeus, 1758 (Caputo 1998); (b) in the telomeric region on the long arm of a single pair of acrocentrics, such as in *Zosterisessor ophiocephalus* (Pallas, 1814) (Caputo 1998); (c) in the interstitial/pericentromeric region on the long arm of a single pair of acrocentric chromosomes, as seen in *Proterorhinus marmoratus* (Pallas, 1814) (Ráb 1985) and *Gobius cobitis* Pallas, 1814 (Caputo 1998); (d) in the telomeric region on the short arm of a single subtelocentric pair, described in *B. soporator*; (e) in the interstitial/pericentromeric region on the long arm of a single metacentric pair, observed in *N. eurycephalus* (Ene 2003); and (f) in the telomeric regions on the short arms of two acrocentric chromosome pairs, recorded in *Gobiusculus flavescens* (Fabricius, 1779) (Klinkhardt 1992).

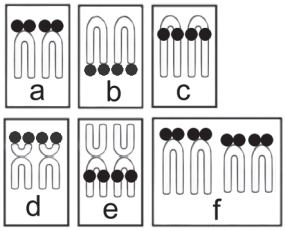

Figure 2. Ag-NOR phenotypes **a–f** described in species of Gobiidae. Ag-NORs sites described in the karyotypes of Gobiidae species were found **a** in the telomeric region on the short arm of a single pair of acrocentric chromosomes **b** in the telomeric region on the long arm of a single pair of acrocentrics **c** in the interstitial/pericentromeric region on the long arm of a single pair of acrocentric chromosomes **d** in the telomeric region on the short arm of a single subtelocentric pair **e** in the interstitial/pericentromeric region on the long arm of a single metacentric pair and **f** in the telomeric regions on the short arms of two acrocentric chromosome pairs.

Few data are available on ribosomal sites for Labrisomidae. Ag-NORs in *L. nuchipinnis* exhibit the phenotype (b) described above, in addition to both species of Blenniidae, *O. trinitatis* and *S. cristata*, which may suggest an ancestral condition for this location.

In contrast, other chromosome characteristics, such as C-positive heterochromatin distribution, may be more conserved. This occurs in several species of Percifomes where discrete blocks are preferentially located in the centromeric/pericentromeric regions of chromosomes (Molina 2007). This pattern is repeated in *S. cristata*, *O. trinitatis* and *L. nuchipinnis*, as well as in some Gobiidae, such as *G. cobitis, Z. ophiocephalus* and *N. eurycephalus* (e.g. Caputo et al. 1997; Ene 2003). In *B. soporator*, in addition to centromeric/pericentromeric regions, heterochromatic sites are also observed in terminal regions of some chromosomes. This arrangement has already been described for other Gobiidae, including *G. paganellus* and *G. niger*, where pericentromeric and telomeric heterochromatic regions are distributed among almost all chromosomes (Amores et al. 1990; Caputo et al. 1997).

Moreover, karyotypic diversity present in Gobioidei is increased by the occurrence of chromosome polymorphisms frequently observed in this group. This is particularly evident in several examples of intraspecific karyotypic variability, as well as polymorphisms involving different types of chromosome rearrangements, such as in *G. niger* (Vitturi and Catalano 1989; Caputo et al. 1997) and *G. fallax* (Thode et al. 1988). Data obtained for the paedomorphic Gobiidae *Aphia minuta* (Risso, 1810) also show variations in the diploid number and chromosome formula, resulting in five different cytotypes (2n=41–44 and FN=42-44) (Caputo et al. 1999). Similar karyotypic variability was reported in *N. eurycephalus,* where three specific cytotypes (2n=30, 31 and 32) were associated to the occurrence of centric fusions (Ene 2003). All these examples demonstrate clear chromosomal dynamism, with possible transitions to new karyotype patterns.

In fact, karyotypic diversity among Blennioidei and Gobioidei seems to accompany phyletic diversification of these groups. This is a result of vicariant factors (Pampoulie et al. 2004) and could be favored by their low dispersive potential (Fanta 1997), as well as ecological specificities that favor population fractionation in this family (Huyse et al. 2004). The present study also highlight the importance of ribosomal sites as effective chromosomal markers in the further cytogenetic studies in gobiids species.

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RESEARCH ARTICLE

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Karyological studies in ten species of *Citrus* (Linnaeus, 1753) (Rutaceae) of North-East India

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Abstract

Ten *Citrus* (Linnaeus, 1753) species of North-East India have been karyo-morphologically analysed. All studied species had 2n=18 chromosomes without any evidence of numerical variation. All the chromosomes were found to be of metacentric and sub-metacentric in all the species; the morphology of the chromosomes showing size difference only. Symmetrical karyotype which does not have much difference in the ratio of longest to shortest chromosome in all the species was observed. Three species, *C. grandis* (Osbeck, 1757), *C. reticulata* (Blanco, 1837) and *C. medica* (Linnaeus, 1753) are identified as true basic species from asymmetry studies of karyotypes as they reflect on the primitive nature of their genomes. *C. indica* (Tanaka, 1937) occupies a special taxonomic position within the genus *Citrus* as a progenitor for other cultivated species.

Keywords

Citrus, karyotype, genetic variability, asymmetry index

Introduction

The genus *Citrus* is economically very important and is known for its juice and pulp throughout the world. The genus belongs to the family Rutaceae that includes 162 species (Tanaka 1977) and is grown in tropical and subtropical areas of the world. *Citrus* is the third most important fruit crop of India with an estimated production of 4.2 million tons from an area of 0.48 m ha (Bathla et al. 2001). Mandarin (*Citrus reticu-*

lata Blanco, 1837), sweet orange (*C. sinensis* Osbeck, 1757), acid lime (*C. aurantifolia* Swingle, 1913) and lemon (*C. limon* Osbeck, 1765) are the major cultivated species of the country. Other species that are cultivated to a lesser extent include seedless lime (*C. latifolia* Tanaka, 1937), pummelo (*C. grandis* Osbeck, 1757), grapefruit (*C. paradisi* Macfadyen, 1930) and belladikithuli (*C. maderaspatana* Tanaka, 1937). In India, there are 30 species of *Citrus* (Singh and Chadha 1993) of which at least nine species are available throughout India, while 17 species are confined to North-Eastern India. It is also reported that nine species are found in the southern region of India, six species in the north-western India while a single species is observed in central region of the country (Singh and Chadha 1993). The north-east region of India is known for its rich diversity in *Citrus* germplasm, reflected in 17 species, 52 cultivars and 7 probable natural hybrids which are found in the region (Bhattacharya and Dutta 1956). A recent study on genetic resources of *Citrus* from north-eastern India indicated an increase in the number of species up to 23 besides one subspecies and 68 varieties (Sharma et al. 2004). *Citrus* plants growing in deep forests undisturbed by biotic factors have also been reported from the region, thus bestowing this area with a special status of "treasure house" of *Citrus* germplasm and also highlighted the lack of our knowledge about the same (Sharma et al. 2004).

The south-east Asia, Australia and the intervening island-areas between Australasia and Central Africa and the north-eastern region of India along with neighbouring China (Mc Phee 1967, Swingle and Reece 1967) are thought to be important centres of origin of *Citrus* and related genera. Many *Citrus* species are believed to be endemic to the region. Seven Indian *Citrus* species fall under the category of endangered species which include *C. indica* Tanaka, 1937, *C. macroptera* Montrouzier, 1960, *C. latipes* Tanaka, 1937, *C. assamensis* Dutta et Bhattacharya, 1956, *C. ichangensis* Swingle, 1913, *C. megaloxycarpa* Lushington, 1910 and *C. rugulosa* Tanaka, 1937 (Malik et al. 2006). Two species, *C. indica* and *C. macroptera*, need special and immediate attention for conservation due to their endemism and high degree of threat perception.

South and western hills of Meghalaya in the North-East are reported to have maximum diversity for *C. reticulata, C. grandis, C. limon* and *C. aurantifolia*. These are extensively cultivated for their taste, good pulp and have very high market demand. *C. indica* is supposed to be the most primitive species and perhaps the progenitor of cultivated *Citrus* (Malik et al. 2006) and is locally known as Memang Narang. It is a rare species which is confined to the Tura ranges of West Garo Hills (Upadhyay and Sundriyal 1998). *C. macroptera* is reported to grow in the Khasi and Garo Hills of Meghalaya, North Cachar, Karimganj and Karbi-Anglong districts of Assam and the states of Mizoram, Tripura and Manipur (Bhattacharya and Dutta 1956, Sharma et al. 2004). *C. megaloxycarpa* locally known as 'Sishupal' is a rare species, confined to the Jampui Hill regions of Mizoram and *C. latipes* shows maximum occurrence in West Khasi Hills of Meghalaya.

The relationship between the species within the genus *Citrus* has been made complicated due to combination of factors such as wide cross compatibility, repeated cross pollination and apomixis. Wide hybridization in *Citrus* affects karyotype stability

(Khan 2007). Hybridization has probably played an important role in the evolution of most *Citrus* species. Scora (1975) and Barrett and Rhodes (1976) suggested that there are only three basic species of *Citrus,* that are considered true ones within subgenus *Citrus* while other species within this subgenus are hybrids derived from the three true species or by intercrossing with species of subgenus *Papeda* (Swingle, 1943) or other closely related genera. Wild relatives of cultivated *Citrus* species can be a major source of genetic variation for utilization in breeding programs aimed at crop improvement through transfer of disease resistance or other desirable agronomic traits.

The cytogenetical characterization of *Citrus* accession could help in the identification of a particular genomic variant, or for the detection of true hybrids in breeding program, as well as for studies of karyotypes evolution of the group (Guerra et al. 1997). Despite the great genetic diversity and economic significance attached to several species of *Citrus*, attempts to understand the genetic basis of variation is not forth coming. The available information is scant and fragmented. A quick perusal of the published literature indicates different chromosome number reports in several species such as 2n=18 or 2n=27 in *C. aurantifolia* (Longley 1925; Krug and Bacchi 1943) and 2n=18, 27, 36 in *C. limonia* Osbeck, 1757 (Frost 1925a, b) are case examples. Therefore there is an urgent need to undertake comprehensive cytogenetical approaches to define the existing genetic variation at inter- and intra-specific levels in the genus *Citrus.* The present investigations are an attempt to conduct karyomorphological studies on 10 species of *Citrus* from North-East India.

Material and methods

The plant material used in the present investigation was collected from various region of North-East India and the vouchers specimens have been submitted to National Herbarium of Crop Plants, National Bureau of plant Genetics Resources, New Delhi (Table 1). The plants were grown in green house of Plant Biotechnology Laboratory, Department of Biotechnology and Bioinformatics of North-Eastern Hill University, Shillong. For each species, wherever possible, a minimum of five individuals and more than one population were analyzed. For obtaining actively growing root tips, plants were raised in earthen pots and the root tips of about (0.5–1.0 cm) long were excised. All the root tips were pre-treated with 8-hydroxyquinoline (0.002M) for three hours at room temperature, fixed in ethanol-acetic acid (v/v, 3:1) and subsequently stored at 4 ^{o}C until required. For slide preparation, the root tips were washed twice in distilled water, hydrolysed in 5N HCl for 20 min at room temperature. The hydrolysed root tips were washed in distilled water and stained in Feulgen stain for 45 min. The root tips were subsequently squashed in 1% acetocarmine. The micro-photographs were taken using Jenoptik CCD camera (Germany) attached to labomed LX 400 brightfield microscope. At least five clear preparations of chromosome complements of each species were analyzed for the karyotypes. Idiograms were prepared from photo-micrographs by cutting out individual chromosomes, arranging them in descending order of their

Table 1. *Citrus* species used in the present investigation.

Sl. No.	Species	Common Name	Collection No.	Source
Subgenus Citrus				
1	*C. reticulata*	Khasi Mandrin	CR-9	Pynursla
2	*C. jambhiri*	Rough lemon	CJ-6	Wahkhen
3	*C. sinensis*	Sweet orange	CS-2	Shillong
4	*C. limon*	Assam Lemon	MD/33	Mizoram
5	*C. grandis*	Pummelo	CG-7	Ri Bhoi
6	*C. limetta*	Sweet limes	CLe-1	Shillong
7	*C. indica*	Indian wild orange	SO1	Nokrek, Garo hills
8	*C. medica*	Citron	CMi-2	Wahkhen
Subgenus Papeda				
9	*C. macroptera*	Melanesian Papeda	CMa-1	Cherrapunjee
10	*C. latipes*	Khasi Papeda	Clt-2	Upper Shillong

length and matching on the basis of morphology. The standard method of chromosome classification (Levan et al. 1964) of metacentric (V), submetacentric (L), subtelocentric (J) and telocentric (I) based on the arm ratio of 1:1, >1:1<1:3, >1:3<1:0 and 1:0 respectively, was used for comparison. The degree of symmetry was estimated as per the scheme proposed by Paszko (2006).

Results

The data related to chromosome complements/karyotypes have been presented in Table 2 and illustrated in Fig. 1 and 2 and it is amply clear that among the 10 species of *Citrus* presently studied, two species namely *C. jambhiri* Lushington, 1910 and *C. limon* Linnaeus, 1753 were characteristic in having exclusively sub-metacentric chromosomes in the chromosomes complements. On the other hand the remaining 8 species namely *C. macroptera, C. grandis, C. medica* Linnaeus,1753*, C. reticulata, C. sinensis, C. latipes, C. indica* and *C. limetta* Linnaeus, 1753 had at least one pair of metacentric chromosome among the chromosome complements. It was more intriguing to record that two metacentric pairs were observed in *C. reticulata* and *C. latipes* as metacentrics while one pair of metacentric were recorded in remaining 6 species. Further the position of the meta-centrics varied in different species of *Citrus* presently studied ranging from 2nd pair (in *C. grandis and C. latipes*), 3rd pair (in *C.reticulata*), 4th pair (in *C. macroptera* and *C. indica*), 5th pair (in *C.reticulata, C.latpipes* and *C. limetta*), 7th pair (in *C. sinensis*) and 8th pair (in *C. medica*). Thus, the 6th and 9th pairs in all the species have been found to be invariably sub-metacentric.

Sub-telocentric and telocentric chromosomes which are presumed to significantly influence the symmetry of the karyotype were alltogether absent in any of the species presently studied. From the details of karyotypic formula derived for various species

Table 2. Karyotype formulae and characteristics in 10 species of *Citrus.* AI- asymmetry index; SC - the shortest chromosome length; LC - the longest chromosome length; CL - mean length of chromosome; CI - mean centromeric index; SD - standard deviation; CV_{CL} - component expressing the relative variation in chromosome length; CV_{CI} - component expressing the relative variation in centromeric index.

Species	Collection No	2n	Number of second-dary constriction	Range SC-LC (µm)	Ratio LC/SC	CL (µm) Mean (±SD)	CI Mean (±SD)	CV_{CL}	CV_{CI}	AI	Karyotype formula*
C. macroptera	Cma-1	18	-	5.01–10.52	2.09	7.44 (±1.9)	40.99 (±5.4)	25.53	13.17	3.36	16L+2V
C.grandis	CG-7	18	2	4.03–11.12	2.75	7.83 (±2.04)	43.93 (±3.2)	26.05	7.28	1.89	16L+2V
C.medica	Cmi-1	18	-	4.51–12.02	2.66	6.88 (±1.84)	42.73 (±3.0)	26.74	7.02	1.87	16L+2V
C.reticulata	CR-9	18	-	4.01–9.03	2.25	6.51 (±1.5)	43.07 (±4.6)	23.04	10.68	2.46	14L+4V
C. sinensis	CS-2	18	-	4.03–9.51	2.35	6.71 (±1.34)	43.88 (±6.7)	19.97	15.26	3.04	16L+2V
C. jambhiri	CJ-6	18	-	4.04–9.02	2.23	5.51 (±1.2)	38.72 (±6.7)	21.77	17.3	3.76	18L
C. latipes	CLt-1	18	-	4.02–10.11	2.51	7.08 (±1.76)	39.97 (±6.9)	24.85	17.26	4.28	14L+4V
C. indica	SO1	18	-	4.01–8.14	2.02	5.81 (±1.28)	43.1 (±3.8)	22.03	8.81	1.94	16L+2V
C. limon	MD/33	18	2	4.03–9.10	2.25	6.38 (±1.39)	42.16 (±5)	21.78	11.85	2.58	18L
C. limetta	Cle-1	18	-	3.51–9.01	2.56	6.18 (±1.77)	42.48 (±4.8)	28.64	11.29	3.23	16L+2V

* As per the method of Levan et al 1964

of *Citrus*, three patterns of karyotype formulae, 18L, 16L+2V and 14L+4V, were recorded. The ratio of longest to shortest chromosomes was recorded as highest in *C. grandis* and the lowest in *C. indica*.

Partial homology among the somatic chromosomes is often expressed in the form of heteromorphism and heteromorphic pairs in karyotypes. The present observation of 10 different species of *Citrus* had shown interspecific diversity with regards to presence or absence of heteromorphic pair in the chromosome complements. C. *macroptera, C. reticulata, C. limon* and *C. latipes* were characteristic in lacking any heteromorphic pair, while *C. grandis, C. medica* and *C. limetta* are unique in having two pairs of heteromorphic chromosomes in their respective complements. One pair of heteromorphic chromosomes was characteristic in *C. sinensis, C. jambhiri* and *C. indica.*

Due to technical problems nucleolar chromosome could not be clearly scored in any of the species presently studied, although there were some indications to suggest that the second pair in *C. grandis* and third pair in *C. limon* are probably nucleolar in nature by revealing the secondary constriction.

The asymmetry index (AI) value which has been derived from the data related to Chromosome length (CL) and Centromeric index (along with the co-efficient of variation) has resolved the ten species of *Citrus* presently investigated into two groups, one

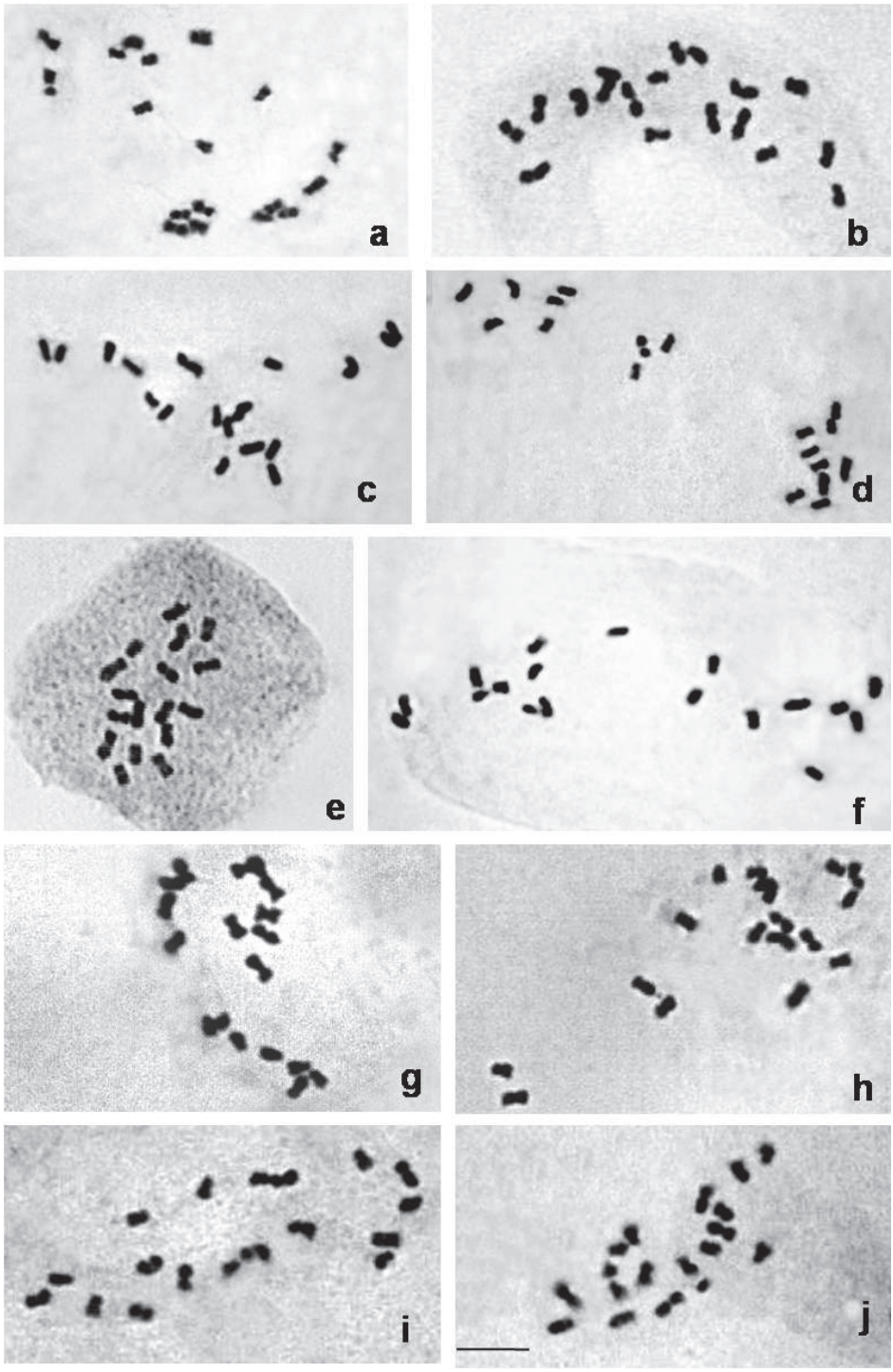

Figure 1. Mitotic complements of 10 *Citrus* species (2n=2x=18). **a** *C. macroptera*, **b** *C.grandis*, **c** *C. medica*, **d** *C. reticulata*, **e** *C. sinensis*, **f** *C. jambhiri,* **g** *C. latipes*, **h** *C. indica,* **i** *C. limon*, **j** *C. limetta.* Bar = 5μm.

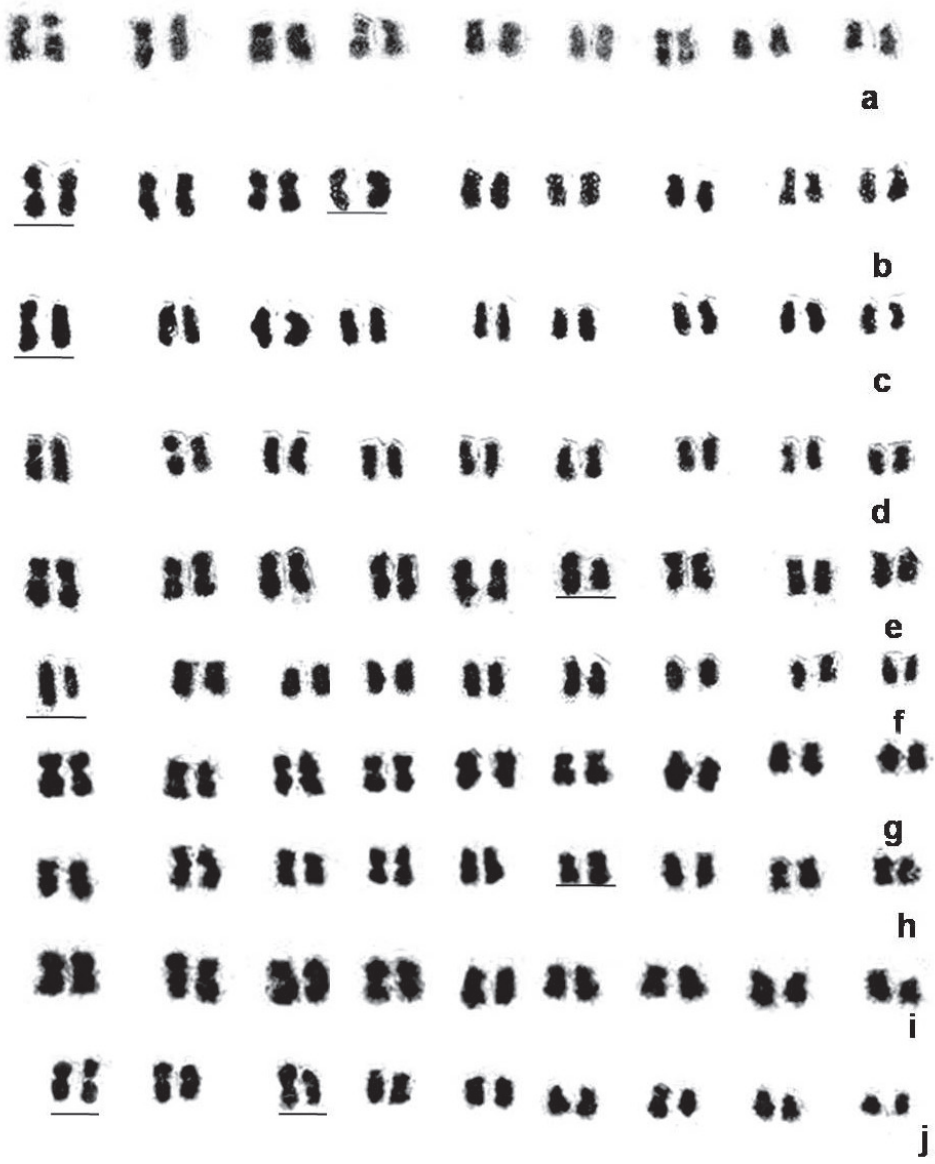

Figure 2. Karyograms of 10 *Citrus* species. **a** *C. macroptera*, **b** *C. grandis*, **c** *C. medica,* **d** *C. reticulata*, **e** *C. sinensis,* **f** *C. jambhiri,* **g** *C. latipes,* **h** *C. indica,* **i** *C. limon,* **j** *C.limetta.* Bar represent heteromorphic pairs.

with low value of asymmetry index indicating high karyotype symmetry corresponding to *C. medica* (1.87), *C. grandis* (1.89), *C. indica* (1.94), *C. reticulata* (2.46). The other group with high asymmetry index indicate low karyotype symmetry corresponding to *C. sinensis* (3.04), *C. limetta* (3.23), *C. macroptera* (3.36), *C. jambhiri* (3.76) and *C. latipes* (4.28). *C. limon* reported to be an intermediate species had an asymmetry index value of 2.58 indicating its link between the above two groups.

Discussion

From the perusal of published literature it can be seen that the somatic chromosome number in the genus *Citrus* is diverse ranging from 2n=18, 27, 36, 54, etc. (Bacchi 1940; Krug 1943; Krug and Bacchi 1943; Lapin 1937) in various species. It can be seen from the above published data, that the relationship is indicative of a probable polyploid series with a basic number of x=9. In the present investigation all the somatic cells analysed in 10 different species had 2n=18. However in one specimen of *C. reticulata* 2n=36 was recorded. Thus the present studies involving 10 representative species did conform the somatic chromosome number as 2n=18 only without any exception.

Thus the present data as reflected from Fig. 1 and 2, combined with chromosome counts available from the literature confirms that the genus *Citrus* is apparently monobasic in nature and x=9 is the most acceptable number. Such observation received an ample support from reports of Krug (1943), Tanaka (1930), Yamamoto et al. (2007), and Barros e Silva et al. (2010). The sporadic occurrence of 2n=36 in a few cells of *C. reticulata* is another indication for x=9 as the true basic number of the genus *Citrus*. The basic chromosome number of *Citrus* (Rutaceae) and other related genera of the subfamily Aurantioideae has been reported as x=9 (Frost 1925). The majority of the wild and cultivated forms of *Citrus* are identified as diploids, i.e. 2n=2x=18 (Krug 1943). However polyploids are known to exist, which arise either spontaneously or following certain cross combination. For example there have been reports of naturally occurring tetraploids from inter-specific crosses between tetraploid and diploid taxa (Oiyama et al. 1991) and induced polyploids by colchicine (Barret 1974, Oiyama and Okudai 1986). Heteroploid crosses involving tetraploid (4x) and diploid (2x) species resulted in spontaneous production of a triploid 'Tahiti lime' (Krug and Bacchi 1943; Oiyama et al. 1991, 1980). Luss (1935) was the first to report about a hypertriploid (3x +1=28). Similar observations of hypertriploid were also reported by Lapin (1937), Krug and Bacchi (1943) who have recorded the occurrence of aneuploid from the progeny of various crosses among diploid species. Inter-specific hybridization, ploidy level and the mono/polyembryonic nature of the *Citrus* variety may also contribute to the frequency of polyploid progenies (Cameron and Soost 1969; Wakana et al. 1981).

In the present studies on 10 different *Citrus* species, the chromosome complements were all resolved into either metacentric or sub-metacentric chromosomes only. From the details of karyotypic formulae derived for these species of *Citrus*, three patterns of karyotype formulae, 18L, 16L+2V and 14L+4V, were recorded and there was complete absence of sub-telocentric and telocentric chromosomes which is indicative of the stability of the genome and of the absence of structural alteration of the chromosomes in the genus *Citrus.* Therefore, it is presumed that speciation in the genus *Citrus* could have been influenced by gene mutations which have no effect in the overall structure of chromosomes.

Swingle and Reece (1967), opined that the genus *Citrus* has only three 'basic' true species viz. Citron (*Citrus medica* L.), Mandarin *(Citrus reticulata* Blanco), and Pummelo (*Citrus grandis* Osbeck), while the rest of the species are hybrid derivatives of any

one of the true species and species belonging to sub genus *Papeda* (Barrett and Rhodes 1976; Federici et al. 1998; Nicolosi et al. 2000; Scora 1975). However the high resolution of karyotypes as observed in the present mitotic preparations does not distinguish between basic true species and derived ones. There was no grouping of chromosomes for distinguishing the karyotypes on the basis of the hybrid nature of species as reported. However, the staining methods used traditionally with aceto-carmine, aceto-orcein or Feulgen's solution were less informative to reveal detailed structure under the usual optical microscope because the mitotic chromosomes are very small (1.0–4.0 μm) and most of them are similar in morphology (Krug 1943). Therefore, to establish the hybrid nature of some of the species can only be determine by using more sensitive technique like *in situ* hybridization and the study of banding patterns of the chromosomes.

From the karyological data presented in Table 2 it can be observed that the asymmetry index of different species of *Citrus* presently investigated had shown significant variation. *C. medica, C. grandis* and *C. reticulata* which are considered as true basic species (Swingle and Reece 1967) are characteristic in having low asymmetry index of 1.87, 1.89 and 2.46 respectively. On the other hand 6 species had higher asymmetry index while *C. indica* had an intermediate value. The lower asymmetry indexes of the 3 species recorded suggest an ancestral genome which makes them as true basic species. The higher asymmetry index value recorded in 6 species is indicative of the fact that their genomes are relatively advanced and are in a process of reorganisation through chromosome structural alterations. *C. indica* with its intermediate value of asymmetry index may be regarded as one of the progenitor species of cultivated *Citrus* (Malik et al. 2006) and has a special position in the genus.

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RESEARCH ARTICLE

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Chromosomal diversification in ribosomal DNA sites in *Ancistrus* Kner, 1854 (Loricariidae, Ancistrini) from three hydrographic basins of Mato Grosso, Brazil

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Abstract

Populations of seven *Ancistrus* species were analyzed from streams and rivers of three hydrographic Brazilian basins. All populations showed different diploid numbers (2n), fundamental numbers (FNs), and karyotypes. Some representatives of Loricariidae have 2n = 54 chromosomes, which is very likely an ancestral cytotaxonomic characteristic, but many other representatives show extensive karyotype diversification. In the *Ancistrus* species studied, extensive karyotypic differentiation, which is generally associated with chromosome number reduction and rearrangement of the ribosomal RNA gene (rDNA) sites, was verified. Chromosomal locations of 18S and 5S rDNA were jointly detected using fluorescence *in situ* hybridization (FISH). In all the *Ancistrus* species analyzed, 18S rDNA sites were detected only on one chromosome pair, though this differed among species. 5S rDNA was located on 1–3 chromosome pairs either separately or in synteny with 18S rDNA in four of the seven species/populations. Hence the karyotype differentiation in *Ancistrus* species could be associated with a morphological speciation process, suggesting that chromosome fusions, inversions, deletions, duplications, and heterochromatination could contribute to the karyotype evolution of these neotropical armored catfishes.

Keywords

karyotype evolution, Robertsonian rearrangement, Ag-NORs, 18S rDNA, 5S rDNA

Introduction

In eukaryotes, 5S and 18S ribosomal genes (rDNA) are arranged into two distinct classes, namely the major rDNA family composed of 18S, 5.8S, and 28S genes and the minor family composed of 5S genes (Long and David 1980, Pendás et al. 1994). Silver nitrate-stained nucleolus organizing regions (Ag-NORs) have long been used in cytotaxonomic analysis of fish (Galetti 1998). However, cytogenetic comparisons of banding patterns prove inadequate when dealing with species with highly rearranged genomes or with other highly divergent species (Chowdhary and Raudsepp 2001). Hence, comparison using fluorescence *in situ* hybridization (FISH) associated with classical chromosomal markers has become the preferred method for genome comparisons at the cytogenetic level because it allows complete chromosome probes of a species to be hybridized *in situ* with chromosomes of other species, thereby allowing the detection of homologous genomic regions (Vicari et al. 2010; Bellafronte et al. 2011; Machado et al. 2011).

Studies that characterize the chromosomal locations of 5S and 18S rDNA in Siluriformes are scarce (Kavalco et al. 2004; Centofante et al. 2006; Mendes-Neto et al. 2011). A few such studies were conducted in Pimelodidade and Pseudopimelodidae, where non-syntenic 5S and 18S ribosomal regions were observed (Carvalho and Dias 2007; Garcia and Moreira-Filho 2008; Marques et al. 2008; Matoso et al. 2011; Moraes Neto et al. 2011; Silva et al. 2011). In Loricariidae, chromosomes with syntenic 5S and 18S regions were observed in some groups like Neoplecostominae and the out group Trichomycteridae (Ziemniczak 2011).

The classification of subfamilies within Loricariidae and the genera relationships have been targets of repeated reformulation (Isbrücker 1980; Armbruster 2004; Reis et al. 2006). Armbruster (2004) placed the subfamilies Hypoptopomatinae, Hypostominae, Lithogeneinae, Loricariinae, and Neoplecostominae as valid groups within this family. In this revision, the former subfamily Ancistrinae is regarded as a synonym of Hypostominae, which comprises five tribes, namely Corymbophanini, Rhinelepini, Hypostomini, Pterygoplichthini, and Ancistrini. The available karyotypic data on Loricariidae show a high diversity of diploid numbers (2n) and chromosomal features, although some evolutionary trends can be defined among the distinct subfamilies (Ziemniczak 2011). Artoni and Bertollo (2001) stated that 2n = 54 chromosomes would be a putative plesiomorphic trait in Loricariidae once it is reported in the basal genera and the sister group of the superfamily Loricarioidea, as described in Trichomycteridae. Therefore, groups such as Loricariinae and Hypostominae present a wide diversity of 2n and chromosomal markers, comprising highly differentiated traits in relation to primitive features of Loricariidae (Artoni and Bertollo 2001; Mariotto et al. 2009). These inferences based on cytogenetic data are in agreement with the last hypotheses of morphological (Armbruster 2004; Reis et al. 2006) and molecular phylogenies (Cramer et al. 2011) in this family.

Among Hypostominae, few species maintain the number of 54 chromosomes. All representatives in Ancistrini have 2n ≤ 54, indicating that centric fusions contributed

to the karyoevolution of this tribe. In this study, we used comparative chromosomal markers to establish chromosome homologies among some *Ancistrus* species and investigated the cytotaxonomical, biogeographical, and karyoevolutionary features of this group.

Materials and methods

One hundred and thirty six specimens [male (M) and female (F)] of seven *Ancistrus* species were cytogenetically analyzed. All specimens were from rivers and streams of three hydrographic basins (a, b, and c) of Mato Grosso state, Brazil: (a) Paraguay basin, Coxipó river, 15°21'59"S, 55°57'11"W, *Ancistrus claro* Knaack, 1999 (11 M and 10 F); Sepotuba river, 14°41'35"S, 57°48'14"W, *Ancistrus* sp. 04 (12 M and 15 F); Currupira river, 15°07'59"S, 56°49'47"W, *Ancistrus* sp. 08 (7 M and 8 F); Flechas stream, 15°58'7"S, 57°19'7"W, Fundo stream, 16°14'17"S, 56°37'31"W, and Pari stream, 15°36'6"S, 56°12'19"W, *Ancistrus* cf. *dubius* Eigenmann and Eigenmann, 1889 (2 M and 2 F from each locality); Arrombado bay, 16°21'21"S, 56°27'55"W, *Ancistrus cuiabae* Knaack, 1999 (15 M and 15 F); (b) Araguaia–Tocantins basin, Salgadinho stream, 14°40'14"S, 52°21'50"W, *Ancistrus* sp. 13 (11 M and 6 F); and (**c**) Amazon basin, Matrixã river, 10°3'7"S, 57°36'27"W, *Ancistrus* sp. 06 (9 M and 5 F).

Specimens were morphologically identified and deposited in the Museu de Ciências da Pontíficia Universidade Católica do Rio Grande do Sul (MCP/PUC; MCP 41966, 41968, 41971, 41973, 41975, 41978, 41979) and Núcleo de Pesquisas Limnológicas da Universidade Estadual de Maringá, Paraná (NUPELIA/UEM; NUP 6827, 7492). *Ancistrus* sp. 04, 06, 08, and 13 present discriminative morphological characteristics that have not yet been described.

Chromosomal preparations were obtained from anterior kidney cells using an *in vivo* treatment with colchicine (Bertollo et al. 1978). The nucleolar organizing regions (NORs) were located using colloidal silver nitrate (Howell and Black 1980). A digital camera with an 8.1 Mp resolution was used in light field microscopy to photograph the Ag-NORs.

FISH was performed according to Pinkel et al. (1986). Two probes were used, namely an 18S rDNA probe obtained from the nuclear DNA of *Prochilodus argenteus* Spix and Agassiz, 1829 (Hatanaka and Galetti 2004) and a 5S rDNA probe obtained from the genomic DNA of *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti 1999). The 18S and 5S rDNA probes were labeled with biotin-16-dUTP and digoxigenin-11-dUTP, respectively, through nick translation according to the manufacturer's instructions (Roche Applied Science). The overall hybridization procedure was performed under high-stringency conditions (2.5 ng/μL from each probe, 50% deionized formamide, 10% dextran sulfate, 2×SSC, pH 7.0–7.2, incubation at 37°C overnight). After hybridization, the slides were washed in 15% formamide/0.2×SSC at 42°C for 20 min, 0.1×SSC at 60°C for 15 min, and 4×SSC/0.05% Tween at room temperature for 10 min, with the latter con-

sisting of two washes of 5 min each. Signal detection was performed for 1 h using conjugated avidin–fluorescein isothiocyanate (Sigma) for the 18S rDNA and anti-digoxigenin–rhodamine (Roche Applied Science) for 5S rDNA at 1:1000 and 1:200 dilutions, respectively, in non-fat dry milk buffer (5% non-fat dry milk in 4×SSC). The chromosomes were counterstained with DAPI and analyzed under an epifluorescence microscope (Olympus BX41) coupled to an image capturing system (Olympus DP71). Approximately 30 metaphases were analyzed to determine the 2n, karyotypic formulae, and the presence or absence of rDNA sites on the chromosomes.

Results

The studied species showed variations in 2n and in karyotypic formulae (Table 1, Fig. 1). The 2n ranged from 54 chromosomes in *A. claro* to 34 chromosomes in *A. cuiabae*. The fundamental number (FN) varied from 68 to 86 chromosome arms (Table 1). NORs were seen in a single chromosome pair in all the *Ancistrus* sp. analyzed using silver nitrate staining and FISH with 18S rDNA probe (Fig. 1). However, an interspecific variation was observed in the NOR-bearing chromosome pairs and NOR locations in these chromosomes (Table 1, Fig. 1 and 2).

Diploid number (2n), metacentric (m), submetacentric (sm), subtelocentric (st), acrocentric (a), fundamental number (FN), sex chromosome system (SC).

The number of 5S rDNA sites varied among species (Fig. 1). Multiple locations were observed in all species, except *Ancistrus* sp. 06 (Fig. 1). Dual-color FISH using 5S and 18S probes showed co-localized sites on apparently homeologous chromosome pairs in *A. claro*, *Ancistrus* sp. 08, *A.* cf. *dubius*, and *Ancistrus* sp. 06 (Fig. 1). However, in *Ancistrus* sp. 04, *A. cuiabae*, and *Ancistrus* sp. 13, 18S and 5S rDNA probing revealed different chromosome pairs carrying 18S and 5S rDNA (Fig. 1). *Ancistrus claro* showed syntenic rDNA site in pair 21 and four additional 5S rDNA sites (Fig. 1). In *Ancistrus* sp. 04, syntenic 18S and 5S rDNA classes in pair 22 and additional 5S rDNA sites in pairs 17 and 25, as well as one homologue in pair 26 were visualized (Fig. 1). *Ancistrus* sp. 08 showed 18S rDNA syntenic to 5S rDNA in pair 13 and an additional 5S rDNA site in pair 1 (Fig. 1). In addition, *Ancistrus* sp. 08 had a heteromorphic ZZ/ZW sex chromosome system in pair 20 (Fig. 1). *Ancistrus* cf. *dubius* showed syntenic 18S and 5S rDNA classes in pair 16, and additional 5S rDNA sites in pairs 4 and 14 (Fig. 1). This species presented an extensive heterochromatic region in pair 19 of females and in one of the complements of pair 19 of males, thus characterizing a sexual chromosomal system, XX/XY (data not shown). *Ancistrus cuiabae* showed 18S rDNA located in pair 2 and three different pairs (3, 6, and 9) carrying 5S rDNA sites (Fig. 1). In *Ancistrus* sp. 13, 18S rDNA was located in pair 18, and 5S rDNA was located in pairs 5 and 15 (Fig. 1). *Ancistrus* sp. 06 showed syntenic rDNA classes in pair 21 (Fig. 1).

Table 1. Chromosomal data in analyzed *Ancistrus* species.

Species/basin	2n	Karyotypic formulae	FN	SC	rDNA synteny
nParaguay basi					
A. claro	54	14m+8sm+8st+24a	84	-	Present
Ancistrus sp. 04	52	16m+8sm+6st+22a	82	-	Absent
Ancistrus sp. 08	44	18m+10sm+8st+8a	80	ZZ/ZW	Present
A. cf. *dubius*	42	24m+10sm+8st	84	XX/XY	Present
A. cuiabae	34	20m+8sm+6st	68	-	Absent
nAraguaia–Tocantins basi					
Ancistrus sp. 13	40	26 m+10sm+4st	80	-	Absent
nAmazon basi					
Ancistrus sp. 06	50	18m+10sm+8st+14a	86	-	Present

Discussion

The catfish Loricariidae is one of the most speciose components of neotropical freshwater fish fauna. The karyotypic differentiation of Ancistrini is correlated with the great diversification of forms in this tribe and may play an important role in the genetic/reproductive isolation of species (Ziemniczak 2011). Some representatives of Loricariidae have 2n = 54 chromosomes, which is very likely an ancestral cytotaxonomic characteristic, but many other representatives show extensive karyotype diversification (Artoni and Bertollo 2001; Milhomem et al. 2010; Bueno et al. 2011; Ziemniczak 2011). *Ancistrus* is the most speciose genus in the tribe and exhibits extensive karyotypic differentiation, generally associated with a chromosome number reduction (Alves et al. 2003; Mariotto et al. 2009).

This study revealed that chromosome fusion is the major mechanism in 2n reduction of some Ancistrini species. In the sister group Hypostomini, in which all species present 2n ≥ 54, it has been postulated that the increase in the subtelocentric/acrocentric chromosome number is directly proportional to 2n, thereby indicating that centric fissions have played a key role in karyotype evolution of the group (Artoni and Bertollo 2001; Milhomem et al. 2010). Although this hypothesis is partially supported in this tribe, it was not possible to correlate 2n with the proportion of subtelocentric/acrocentric chromosomes in some species (Bueno et al. 2011). In this study, we hypothesized that the 2n primitive to the family is conserved in *A. claro*, and extensive chromosomal rearrangements, such as chromosome fusions, inversions, deletions, duplications, and heterochromatination, could contribute to the chromosomal differentiation of Ancistrini. This assumption is corroborated by the NF value (Table 1), which is not maintained solely by chromosome fusion.

Ag-NORs can also be used as efficient markers in Loricariidae. A single NOR pair in an interstitial location is considered a primitive characteristic in Loricariidae and is maintained in most Ancistrini species (Artoni and Bertollo 2001; Alves et al. 2003; Mariotto et al. 2004; Mariotto and Miyazawa 2006; de Oliveira et al. 2006, 2007,

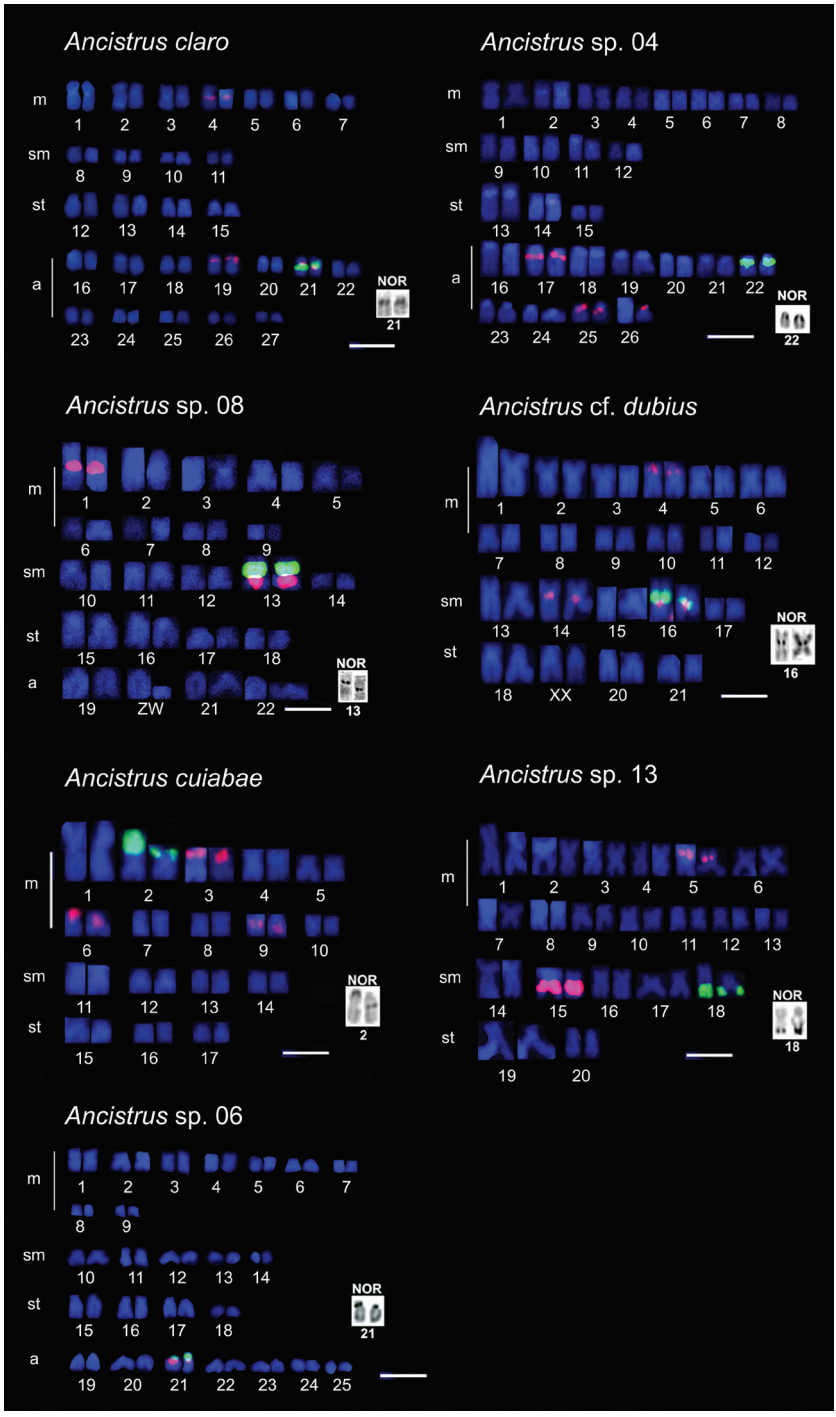

Figure 1. Chromosomes of *Ancistrus* species after dual color-FISH showing 5S rDNA (red) and 18S rDNA (green) sites. Silver nitrate-stained nucleolar organizing region (Ag-NOR) patterns are shown in the boxes. Bars = 10 μm.

Mariotto et al. 2009; Mendes Neto et al. 2011). Utilizing rDNA probes for dual-color FISH experiments have provided important information about the chromosomal diversification of this fish group. Ziemniczak (2011) described syntenic, adjacent, and interstitial locations of 18S and 5S rDNA classes in a single chromosome pair in the basal genera of Loricariidae and in its out group, Trichomycteridae. Based on these data, Ziemniczak (2011) inferred that the synteny between both rDNA classes in a single chromosome pair is a primitive condition for Loricariidae.

Ancistrus claro, *A.* cf. *dubius*, *Ancistrus* sp. 08, and *Ancistrus* sp. 06 conserve the interstitial NORs in a putative homologous pair. However, the chromosomal morphologies of NOR-bearing chromosomes vary, probably because of the accumulation of adjacent heterochromatin (Vicari et al. 2006, 2008; Kantek et al. 2009) and/or by variation in the size of rDNA from unequal crossover. The latter mechanism can also explain18S rDNA polymorphism in *A. cuiabae*. Based on these data, it can be said that the proposal of an ancestral karyotype in the genus is similar to that presented by *A. claro*, which has 2n = 54 chromosomes, a high NF value, and one chromosome pair with syntenic 5S and 18S rDNA classes.

FISH mapping of 5S rDNA in *Ancistrus* species showed variations in the number and shape of chromosomes bearing this ribosomal family. Most sites were observed in the interstitial portion of the long or short arm of chromosomes; however, in some cases, such as *A.* cf. *dubius* and *Ancistrus* sp. 08, pericentromeric 5S rDNA sites were visualized. The occurrence of multiple and variable 5S rDNA can be considered an important process underlying this huge karyotypic diversity. In the subfamily Loricariinae, Ziemniczak (2011) demonstrated that interstitial telomeric sites and 5S rDNA can be observed in fusion chromosomes, implying that rDNA could serve as a breakpoint for fusion in *Rinelocaria lima*. Thus, the mechanism generating fused chromosomes or others by sequence transpositions that promote chromosome diversification can help to explain karyotypic evolution in *Ancistrus* species.

Based on the trends in the karyotype evolution in Ancistrini, it can be inferred that the variation in 2n (54–34 chromosomes) in the *Ancistrus* species studied could possibly involve several chromosomal rearrangements and gene flow restriction in different hydrographic basins or rivers (Fig. 2). The 2n primitive was found in *A. claro* from the Paraguay basin, which presents syntenic 18S and 5S rDNA sites in pair 21 and no sex chromosome heteromorphism. However, this species presents two additional 5S rDNA sites, a characteristic considered apomorphic in this family. *Ancistrus* sp. 04 from the Paraguay basin presents 2n = 52 chromosomes, no syntenic rDNA sites, no sex chromosome heteromorphism, and additional 5S rDNA sites. Hence, rDNA translocation and fusion chromosomes could have occurred in species diversification, and the broken condition of the syntenic rDNAs could have originated in one lineage with *A. cuiabae* and *Ancistrus* sp. 13. The similarity among them is maintained by no syntenic rDNA sites, no sex chromosome heteromorphism, and closely hydrographic basins. However, the pair carrying 18S rDNA in *Ancistrus* sp. 04 is apparently homeologous to chromosome pair 18 in *Ancistrus* sp. 13, which could have originated in the second pair in *A. cuiabae* by chromosome fusion.

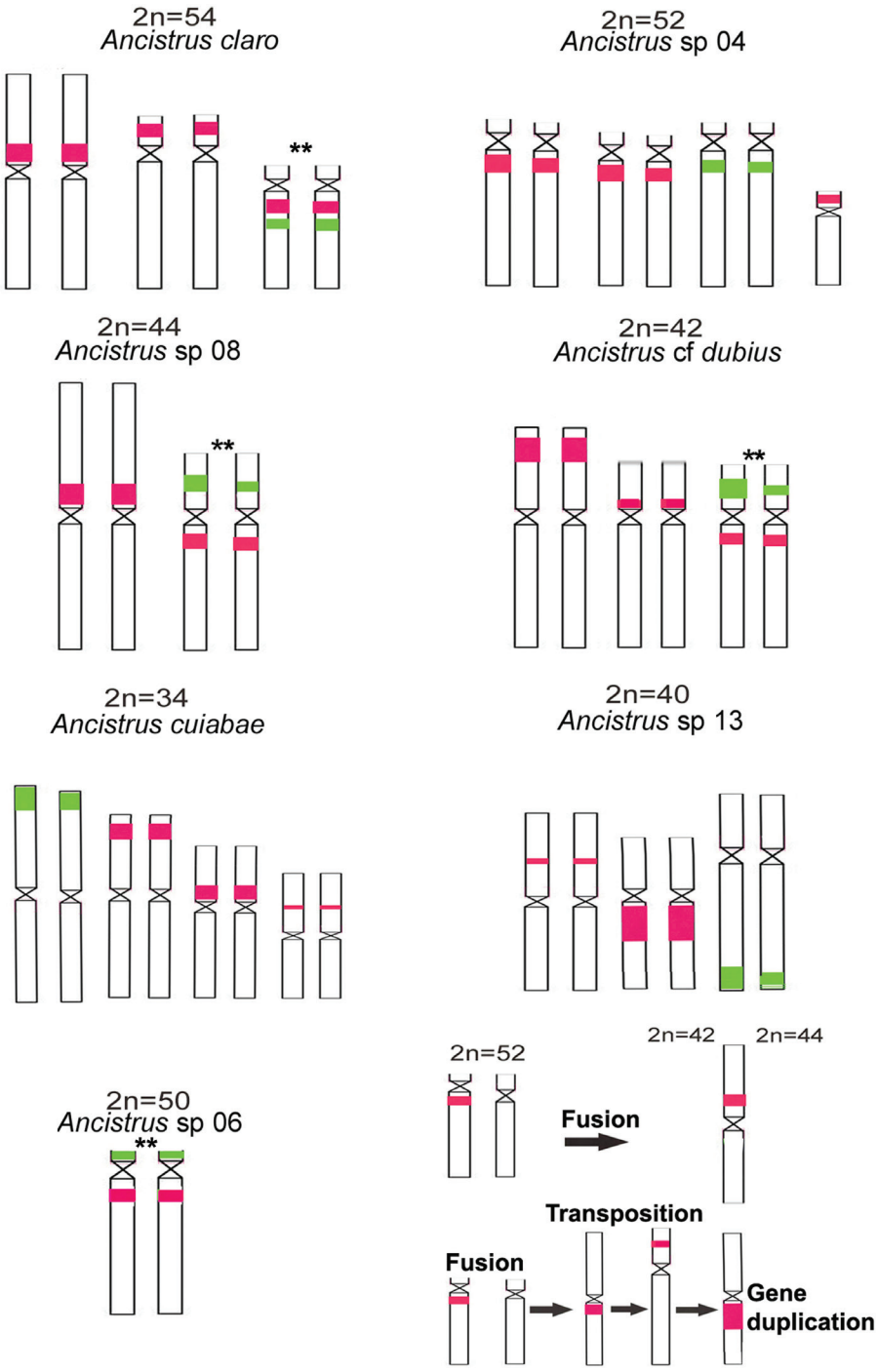

Figure 2. Idiograms of chromosomes bearing 5S (red) and 18S (green) rDNA (a–g); (h) probable chromosomal rearrangements (fusions, transpositions, and gene duplication) occurred during the evolution of *Ancistrus* species; ** denotes possible homeologous chromosomes.

The other lineage consists of species that retain the syntenic rDNA sites (*A. claro*, *Ancistrus* sp. 08, *A.* cf. *dubius*, and *Ancistrus* sp. 06). In addition to *A. claro*, species from the Amazon basin (*Ancistrus* sp. 06) have 2n = 50 chromosomes, considered to have been derived from the family. However, this species retains a primitive single NOR pair with syntenic 5S rDNA site and no additional site. Thus, the chromosome number reduction in *Ancistrus* sp. 06 is attributable to chromosome fusion. *Ancistrus* sp. 08 and *A. dubius* from the Paraguay basin have chromosome number reduction (2n = 44 and 2n = 42, respectively) by fusion and independent pathways to differentiated sex chromosome systems (Mariotto et al. 2004; Mariotto and Miyazawa 2006). *Ancistrus* sp. 08 shows a ZZ/ZW system relative to pair 20 (Mariotto and Miyazawa 2006), whereas *A. dubius* has a XX/XY heteromorphic sex system in pair 19 (Mariotto et al. 2004).

Nevertheless, there is large chromosome plasticity among the species from the Paraguay basin, and the diversity of chromosome types with 5S and 18S ribosomal cistrons in *Ancistrus* sp. explain the high degree of karyotypic diversification in this taxon. Also, the 18S rDNA marker, which is mostly considered to be conserved in a single chromosome pair in the interstitial position, showed different site locations in different types of chromosomes.

The variation observed in the 2n, FN, and rDNA sites of the *Ancistrus* sp. could be attributed to structural and numeric chromosome rearrangements. The karyotypic data presented here are important tools for taxonomy of *Ancistrus* species. The karyotype differentiation in Ancistrini could be associated with a morphological speciation process, suggesting that chromosome fusions, inversions, deletions, duplications, and heterochromatination could contribute to the chromosomal differentiation of Ancistrini.

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B microchromosomes in the family Curimatidae (Characiformes): mitotic and meiotic behavior

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Abstract

In the present work, six curimatid species were analyzed: *Cyphocharax voga* (Hensel, 1870), *C. spilotus* (Vari, 1987), *C. saladensis* (Meinken, 1933), *C. modestus* (Fernández-Yépez, 1948), *Steindachnerina biornata* (Braga & Azpelicueta, 1987) and *S. insculpta* (Fernández-Yépez, 1948) collected from two hydrographic basins. All samples presented 2n=54 meta-submetacentric (m-sm) chromosomes and FN equal to 108, and 1 or 2 B microchromosomes in the mitotic and meiotic cells of the six sampled populations showing inter-and intraindividual variation. The analysis of the meiotic cells in *C. saladensis*, *C. spilotus,* and *C. voga* showed a modal number of 54 chromosomes in the spermatogonial metaphases and 27 bivalents in the pachytene, diplotene, diakinesis and in metaphase I stages, and 27 chromosomes in metaphase II; in *C. modestus, S. biornata,* and *S. insculpta,* spermatogonial metaphases with 54 chromosomes and pachytene and metaphase I with 27 bivalents were observed. The B microchromosome was observed as univalent in the spermatogonial metaphase of *C. spilotus*, in the pachytene stage in the other species, with the exception of *C. saladensis,* and *S. biornata* in metaphase I. New occurrences of the B microchromosome in *C. voga*, *C. saladensis* and *S. biornata* were observed, confirming that the presence of this type of chromosome is a striking characteristic of this group of fish.

Keywords

B microchromosome, meiosis, curimatids

Introduction

B chromosomes, also known as supernumerary or accessory chromosomes, are additional dispensable chromosomes present in some individuals of some populations in some species. They have probably originated from the A complement, but followed their own evolutionary paths, being found in different groups of both animals and plants (Camacho et al. 2000).

The irregular behavior of this chromosome type in mitosis and in meiosis causes it to accumulate selfishly in the germ line of many species, producing a non-Mendelian segregation with transmission rates higher than those yielded by the chromosomes of the A complement (Camacho et al. 2000). B chromosomes present in an individual can exhibit a parasitic, neutral or beneficial behavior (Jones and Rees 1982).

In freshwater Neotropical fish, the occurrence of B chromosomes has been reported in 61 species, distributed in 16 families of seven different orders and in distinct hydrographic basins, according with the revision accomplished by Carvalho et al. (2008). The order Characiformes possesses the majority of the species bearing B chromosomes, including 31 species of six different families: Anostomidae, Characidae, Crenuchidae, Curimatidae, Parodontidae and Prochilodontidae.

The first work to record the presence of the B chromosome in the family Curimatidae was carried out by Venere and Galetti (1985) in an individual of *Cyphocharax modestus* (Fernández-Yépez, 1948) collected from the Tiete River, municipality of Águas de São Pedro/SP, which proved to be entirely heterochromatic. Since then, other populations of *Cyphocharax modestus* and other species, such as *Cyphocharax spilotus* (Vari, 1987), *Cyphocharax gouldingi* Vari, 1992 and *Steindachnerina insculpta* (Fernández-Yépez, 1948) have shown the presence of this extra chromosome (Gravena et al. 2007; Venere et al. 2008).

The current study examines the frequency, behavior and distribution of B microchromosomes in mitotic and meiotic cells in six fish species of the family Curimatidae from two hydrographic basins.

Material and methods

Six species of the family Curimatidae were analysed: *Cyphocharax voga* (Hensel, 1870), *C. spilotus* (Vari, 1987), *C. saladensis* (Meinken, 1933), *C. modestus* (Fernández-Yépez, 1948), *Steindachnerina biornata* (Braga & Azpelicueta, 1987) and *S. insculpta* (Fernández-Yépez, 1948), collected from the Laguna dos Patos Hydrographic System/RS and Paranapanema River basin/SP/PR (Fig. 1, Table 1). Voucher specimens are catalogued in the Zoology Museum of the Universidade Estadual de Londrina, Paraná state, under catalog numbers: MZUEL 5105 – *Cyphocharax voga*; MZUEL 5106 – *C. spilotus*; MZUEL 5058 – *C. saladensis*; MZUEL 1374 – *C. modestus*; MZUEL 5059 – *Steindachnerina biornata* and MZUEL 1042 – *S. insculpta*.

Mitotic chromosomes were obtained by direct preparation removing the anterior kidney, according to Bertollo et al. (1978) and meiotic chromosomes were obtained using gonadal cells by technique developed by Kligerman and Bloom (1977), with modifications. Chromosomes were characterized as metacentric (m) and submetacentric (sm), according to Levan et al. (1964).

Results and discussion

All samples analyzed showed a diploid number of 54 meta-submetacentric chromosomes (m-sm) and a fundamental number (FN) equal to 108 (Fig. 2). This karyotype structure is often found in this fish group, and are conservative among the species of the family Curimatidae, as already observed by Brassesco et al. (2004) and Venere et al. (2008). Among the populations studied, *Cyphocharax voga* and *C. spilotus* collected in Capivara stream/RS and *C. modestus* and *Steindachnerina insculpta* collected in Três Bocas stream/PR are living in sympatry.

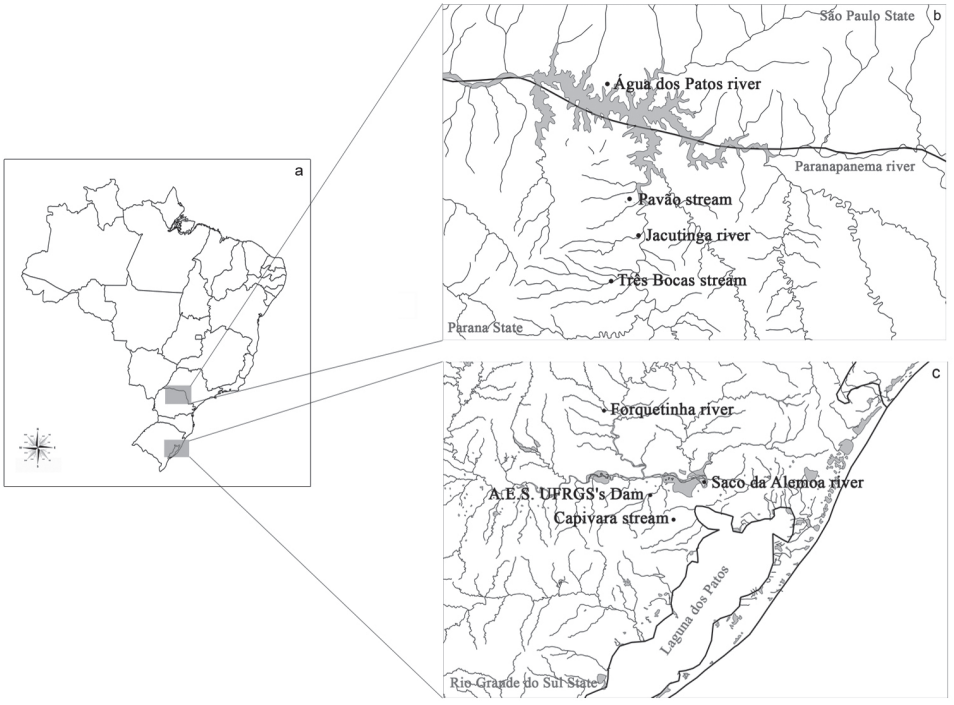

Figure 1. a Map of Brazil **b** Collection sites of Paranapanema River basin: Água dos Patos River in the São Paulo state, Pavão stream, Jacutinga River and Tres Bocas stream in the Parana state **c** Collection sites of Laguna dos Patos Hydrographic System: Forquetinha River, Saco da Alemoa River, Agronomic Experiment Station of UFRGS's Dam and Capivara stream in the Rio Grande do Sul state.

Table 1. Species analysed, collection sites and hydrographic basins.

Species	Number of individuals	Collection sites	Basins
Cyphocharax voga	1♀, 1♂	Saco da Alemoa River, Eldorado do Sul, RS, Brazil S 29°59'15.6", W 51°14'24.1"	Laguna dos Patos Hydrographic System
	2♀, 9♂	Capivara stream, Barra do Ribeiro, RS, Brazil S 30°17'33.3", W 51°19'23.6"	
Cyphocharax spilotus	2♀, 3♂	Capivara stream, Barra do Ribeiro, RS, Brazil S 30°17'33.3", W 51°19'23.6"	
Cyphocharax saladensis	1♀, 10♂	Agronomic Experiment Station of UFRGS's Dam, Eldorado do Sul, RS, Brazil S 30°05'36.2", W 51°40'41.8"	
Steindachnerina biornata	1♀, 1♂	Forquetinha River, Canudos do Vale, RS, Brazil S 29°19'20.9", W 50°14'3.6"	
Cyphocharax modestus	2♀, 5♂	Tres Bocas stream, Londrina, PR, Brazil S 23°17'12.9", W 51°13'58.2"	Paranapanema River
Steindachnerina insculpta	3♂	Tres Bocas stream, Londrina, PR, Brazil S 23°17'12.9", W 51°13'58.2"	
	2♂	Pavão stream, Sertanópolis, PR, Brazil	
	4♀, 8♂	Jacutinga River, Londrina, PR, Brazil S 23°23'6.6", W 51°04'35.8"	
	1♀, 5♂	Água dos Patos River, Iepê, SP, Brazil S 22°41'17.7", W51°05'23.9"	

One B microchromosome was observed in all populations studied, with variation in the number and frequency among them (Fig. 2). In the species *Cyphocharax voga, C. spilotus, C. saladensis* and *Steindachnerina biornata* belonging to the Laguna dos Patos Hydrographic System, there was an inter-and intraindividual variation from 0 to 1 B microchromosome in the somatic cells (Table 2). In *Cyphocharax modestus* and *Steindachnerina insculpta*, from the Paranapanema River basin, up to two B microchromosomes, also exhibiting inter-and intraindividual variation, were detected in the somatic cells (Table 3). As proposed by Jones and Rees (1982), these variations among species represent a mitotic instability of this chromosome, probably due to its non-Mendelian behavior during cell division.

Of the total number of somatic cells with B microchromosomes analyzed in six species of Curimatids, there was a variation from 3.3% in *Cyphocharax saladensis* to 15.4% in *Steindachnerina insculpta*. Among the species belonging to the Laguna dos Patos Hydrographic System, *C. voga* showed the highest percentage of B cells (11.1%), followed by *S. biornata* with 8%, *C. spilotus* with 4.8%, and *C. saladensis* with 3.3% (Table 2).

In the Paranapanema River basin, the population of the *Steindachnerina insculpta* from the Pavão stream/PR showed 15.4% of their somatic cells with B microchromosomes, followed by the populations of the Jacutinga River/PR with 10% and Água dos Patos River/SP with 8.6%. The species *Cyphocharax modestus* from the Tres Bocas stream/PR presented 5% of their cells with B microchromosomes (Table 3). The data

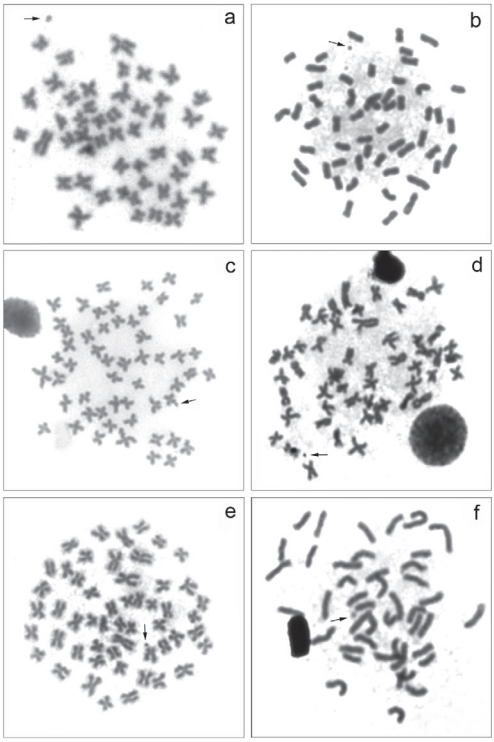

Figure 2. Somatic metaphases: **a** *Cyphocharax voga;* **b** *Cyphocharax spilotus;* **c** *Cyphocharax saladensis;* **d** *Cyphocharax modestus;* **e** *Steindachnerina biornata;* **f** *Steindachnerina insculpta.* The arrows indicate the B microchromosome.

collected from both basins corroborate the constant presence of this type of chromosome in the Curimatidae family, constituting a striking characteristic of the group, even when its incidence is low.

Specimens of *Cyphocharax voga* collected at two localities in the Laguna dos Patos Hydrographic System (Saco da Alemoa River and Capivara stream) not presented interpopulation differences in the number and frequency of the Bs. Likewise were not observed significant differences between the four populations of *Steindachnerina insculpta*, belonging to Paranapanema River basin.

The B microchromosome was observed in four species of curimatids collected from different populations: *Cyphocharax gouldingi* (Venere et al. 2008), *C. modestus* (Gravena et al. 2007), *C. spilotus* (Brassesco et al. 2004), *Steindachnerina insculpta* (Gravena et al. 2007), and three new species assessed in this study: *Cyphocharax saladensis, C. voga* and *Steindachnerina biornata*, representing 18.42% of all species studied, always small in size with inter and intra individual variation (Table 4). Among these, *Cyphocharax modestus* and *Steindachnerina insculpta* are the species that possess B microchromosomes in all populations studied, besides being the species that have the widest range of cytogenetic studies to date.

Table 2. B microchromosome frequency in somatic cells of the curimatids from Laguna dos Patos Hydrographic System/RS.

Species	Locality	Specimens	Sex	Number of B chromosome		Total number of cells
				0	1	
Cyphocharax voga	Saco da Alemoa River	149	♀	22	2	24
		150	♂	3	0	3
		Total		**25**	**2**	**27**
		%		**92,6**	**7,4**	
	Capivara stream	748	♀	3	1	4
		752	♂	4	1	5w
		755	♀	17	0	17
		777	♂	42	1	43
		780	♂	12	0	12
		Total		**78**	**3**	**81**
		%		**96,3**	**3,7**	
Cyphocharax spilotus	Capivara stream	580	♂	2	0	2
		753	♀	25	3	28
		758	♀	23	0	23
		778	♂	8	1	9
		779	♂	22	0	22
		Total		**80**	**4**	**84**
		%		**95,2**	**4,8**	
Cyphocharax saladensis	Agronomic Experiment Station of UFRGS's Dam	784	♂	4	0	4
		786	♀	5	0	5
		787	♂	6	0	6
		788	♂	36	1	37
		789	♂	10	0	10
		790	♂	8	0	8
		791	♂	7	2	9
		792	♂	3	0	3
		793	♂	2	0	2
		794	♂	6	0	6
		Total		**87**	**3**	**90**
		%		**96,7**	**3,3**	
Steindachnerina biornata	Forquetinha River	857	♀	55	3	58
		996	♂	3	2	5
		Total		**58**	**5**	**63**
		%		**92**	**8**	

Camacho et al. (2000), reported that differences in the incidence of B chromosomes among populations depend on selection factors (such as relationship between the Bs and the environmental conditions, including temperature and altitude), historical factors (such as number of generations since the origin of Bs in the population or even in the species), transmission factors (in relation to the mechanisms of accumulation), and random factors. These four types of factors, which are likely to act simultaneously,

Table 3. B microchromosome frequency in somatic cells of the curimatids from Paranapanema River basin.

Species	Locality	Specimens	Sex	Number of B chromosome			Total number of cells
				0	1	2	
Cyphocharax modestus	Tres Bocas stream	2656	♂	5	0	0	5
		3815	♂	18	0	0	18
		3909	♀	8	0	0	8
		3992	♀	46	3	1	50
		Total		**77**	**3**	**1**	**81**
		%		**95**	**3,75**	**1,25**	
Steindachnerina insculpta	Pavão stream	3277	♂	3	2	0	5
		3278	♂	8	0	0	8
		Total		**11**	**2**	**0**	**13**
		%		**84,6**	**15,4**	**0**	
	Água dos Patos River	3393	♀	40	8	0	48
		3407	♂	18	0	0	18
		3408	♂	11	2	1	14
		3409	♂	21	0	0	21
		3411	♂	22	0	0	22
		3745	♂	5	0	0	5
		Total		**117**	**10**	**1**	**128**
		%		**91,4**	**7,8**	**0,8**	
	Jacutinga River	3453	♀	15	2	1	18
		3454	♀	22	0	0	22
		3461	♂	14	0	0	14
		3462	♂	20	0	0	20
		3465	♂	23	1	0	24
		3862	♀	6	0	0	6
		3986	♂	2	0	0	2
		3987	♂	5	0	0	5
		3991	♂	4	0	0	4
		3993	♀	3	0	0	3
		4046	♂	8	0	0	8
		4049	♂	4	10	0	14
		Total		**126**	**13**	**1**	**140**
		%		**90**	**9,3**	**0,7**	

make it difficult to evaluate the action of each one separately, even when a more detailed study of each species occurs.

The analysis of meiotic cells in *Cyphocharax saladensis, C. spilotus* and *C. voga* showed a modal number of 54 chromosomes in spermatogonial metaphases and 27 bivalents in the stages of pachytene, diplotene, diakinesis and metaphase I, and 27 chromosomes in metaphase II (Fig. 3). In *Cyphocharax modestus, Steindachnerina biornata* and *S. insculpta,* spermatogonial metaphases with 54 chromosomes and pachytene and

Table 4. Cytogenetic date of differents species of curimatids (2n: diploid number; FN: number fundamental; Bs: supernumerary chromosomes).

Species	2n	FN	Bs	B Size	References*
Curimata cyprinoides	54	108	-	-	3, 15
Curimata inornata	54	108	-	-	3, 15
Curimata kneri	54	108	-	-	3
Curimata ocellata	56	112	-	-	3
Curimata vittata	54	108	-	-	3
Curimatella alburna	54	108	-	-	3
Curimatella dorsalis	54	108	-	-	8, 12
Curimatella imaculata	54	108	-	-	15
Curimatella lepidura	54	108	-	-	2
Curimatella meyeri	54	108	-	-	3
Curimatopsis myersi	46	46	-	-	8
Cyphocharax gilbert	54	108	-	-	6, 15
Cyphocharax cf. gillii	54	108	-	-	2
Cyphocharax gouldingi	54	108	0 - 1	Micro	15
Cyphocharax modestus	54	108	0 - 4	Micro	1, 2, 7, 9, 13, 14, 16, 17,18
Cyphocharax nagelii	54	108	-	-	2, 15
Cyphocharax cf. spilurus	54	108	-	-	2
Cyphocharax spilotus	54	108	0 - 1	Micro	11, 12, 18
Cyphocharax vanderi	54	108	-	-	2
Cyphocharax voga	54	108	0 - 1	Micro	2, 12, 18
Cyphocharax platanus	58	116	-	-	12, 15
Cyphocharax saladensis	54	108	0 - 1	Micro	18
Potamorhina altamazonica	102	106	-	-	4
Potamorhina latior	56	112	-	-	4
Potamorhina pristigaster	54	108	-	-	4
Potamorhina squamoralevis	102	106	-	-	12
Psectrogaster amazonica	54	108	-	-	15
Psectrogaster curviventris	54	108	-	-	8, 12
Psectrogaster rutiloides	54	108	-	-	3
Steindachnerina amazonica	54	108	-	-	15
Steindachnerina biornata	54	108	0 - 1	Micro	18
Steindachnerina brevipina	54	108	-	-	8, 12
Steindachnerina conspersa	54	108	-	-	2, 12
Steindachnerina elegans	54	108	-	-	2
Steindachnerina gracilis	54	108	-	-	15
Steindachnerina cf. guentheri	54	108	-	-	10
Steindachnerina insculpta	54	108	0 - 2	Micro	2, 5, 13, 14, 15, 17,18
Steindachnerina leucisca	54	108	-	-	3

References: 1 Venere, Galetti (1985) **2** Venere, Galetti (1989) **3** Feldberg et al. (1992) **4** Feldberg et al. (1993) **5** Oliveira, Foresti (1993) **6** Venere, Galetti-Jr (1995) **7** Martins et al. (1996) **8** Navarrete, Júlio-Jr. (1997) **9** Venere et al. (1999) **10** Carvalho et al. (2001) **11** Fenocchio et al. (2003) **12** Brassesco et al. (2004) **13** Gravena et al. (2007) **14** Teribele et al. (2008); **15** Venere et al. (2008) **16** De Rosa et al. (2007) **17** De Rosa-Santos et al. (2008) **18** present study.

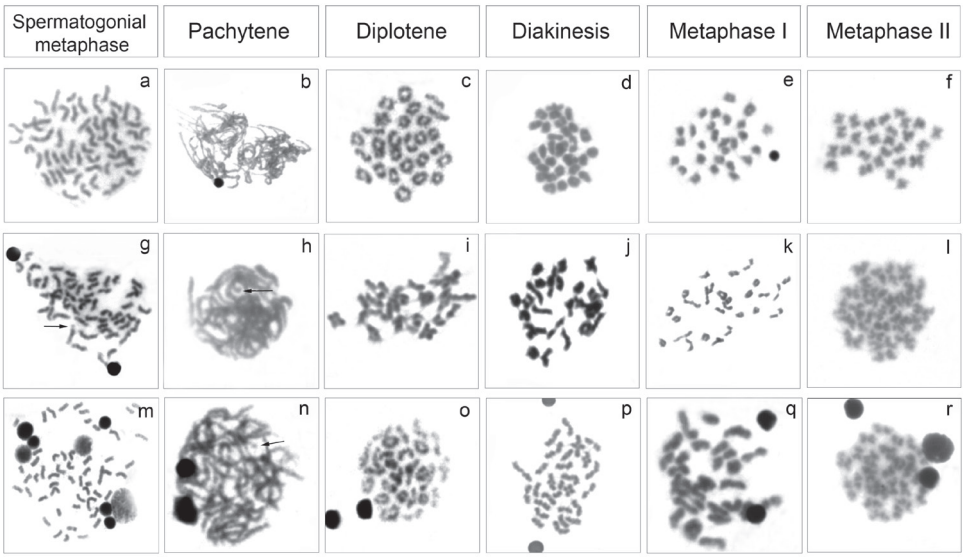

Figure 3. Meiotic cells: *Cyphocharax saladensis* **a–f** *Cyphocharax spilotus* **g–l** and *Cyphocharax voga* **m–r** belonging to Laguna dos Patos Hydrographic System. The arrows indicate the B microchromosome univalent in **g, h** and **n**.

metaphase I with 27 bivalents were observed (Fig. 4). It was possible to observe the B microchromosome as univalent in the spermatogonial metaphase in *Cyphocharax spilotus;* in the pachytene stage in *C. spilotus, C. voga, C. modestus, Steindachnerina biornata* and *S. insculp*ta; and in metaphase I in *S. biornata* (Figs 3, 4).

In both types of cell division, the number of cells without B microchromosomes was greater than number of cells with B microchromosomes in the species of Curimatidae. Camacho et al. (2000) suggest that the small number of chromosomes in diploid cells represents the maximum that a species is able to tolerate as adults.

In others groups of fishes with B-chromosomes meiotic analysis has been performed in order to understand the behavior of this chromosome, as in *Prochilodus lineatus* (Valenciennes, 1836) from the Mogi Guaçu River (Pirassununga/SP), whose studies of the synaptonemal complex showed that no B chromosome paired with autosomal chromosomes. In the late pachytene stage, 27 paired bivalents and small bivalent, trivalent and quadrivalent B chromosomes were observed. The pairing of B chromosomes was interpreted as a result of homology between these chromosomes (Dias et al. 1998).

Borin and Martins-Santos (2004) analyzed *Pimelodus sp.* and *P. ortmanni* Haseman, 1911 from the Iguaçu River, in the Parana state, which had 2n=56 and intraindividual variations from 0 to 4 B chromosomes in the somatic cells. The meiotic analysis confirmed the presence of these chromosomes, with a variation ranging from 0 to 2 B chromosomes in metaphase I, but could not confirm whether these Bs were univalent or bivalent. The species *Rineloricaria pentamaculata* Langeani & Araujo, 1994 from the Tauá stream, Parana River basin, studied by Porto et al. (2010), also

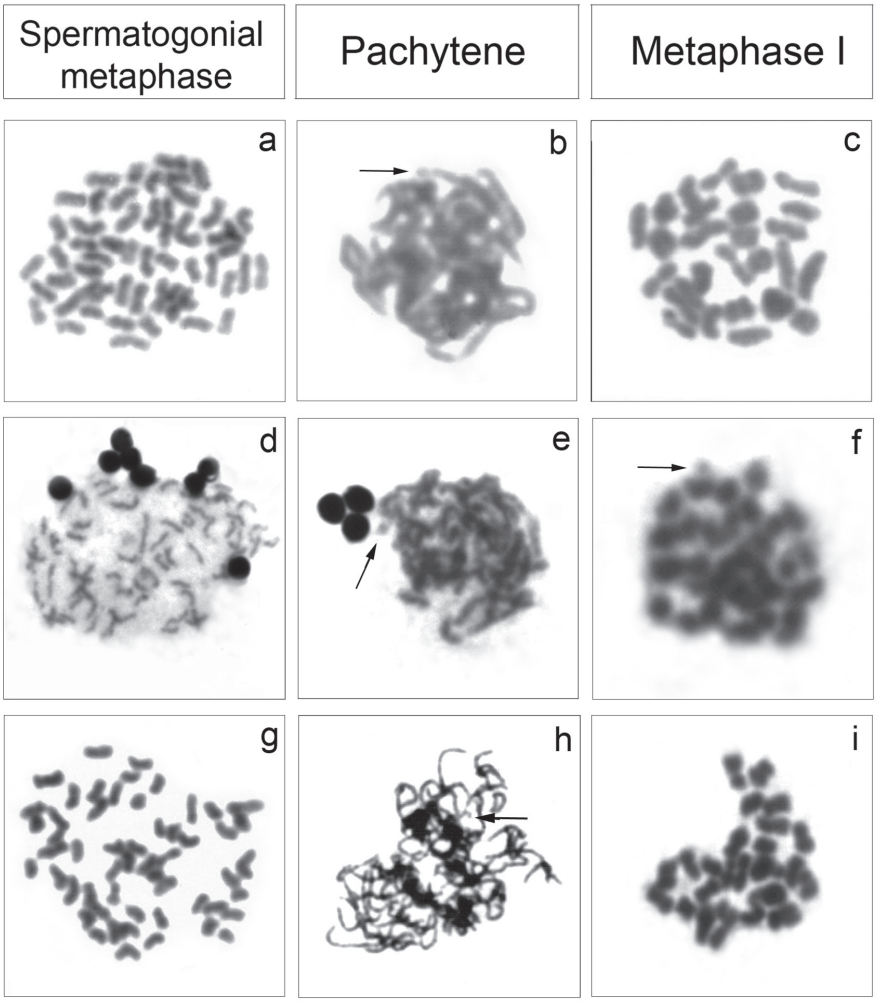

Figure 4. Meiotic cells: *Cyphocharax modestus* **a–c**, *Steindachnerina biornata* **d–f** and *Steindachnerina insculpta* **g–i**. The arrows indicate the B microchromosome univalent in **b, e, f** and **h**.

showed a variation in the diploid number from 56 to 59 chromosomes, attributed to the presence of B chromosomes, which ranged from 0 to 3 in the somatic cells, and confirmed by the meiotic analysis that showed 28 bivalents in metaphases I and II and small univalents. These data support the classification of such elements as supernumerary or B chromosomes, indicating meiotic instability in the transmission to the offspring (Porto et al. 2010).

The meiotic data presented in this study are the first for Curimatidae, and also indicate the instability of the B microchromosome during meiosis, demonstrating that this chromosome has no homology with any normal chromosome complement in these species. Analyses of the synaptonemal complex in the analyzed species would be

interesting to complement the study of the meiotic behavior of B microchromosome in the species Curimatidae.

According Camacho et al. (2000), these chromosomes could be originated from the A chromosomes (intraspecific origin) or as result of mating between species (interspecific origin). Some authors discuss the origin of the B chromosomes in different species of fish as in *Astyanax scabripinnis* (Jenyns 1842) (Moreira-Filho et al. 2004), Amazon species cichlids (Feldberg et al. 2004) and *Rhamdia quelen* (Quoy & Gaimard, 1824) (Moraes et al. 2009).

There are two hypotheses that could explain the origin of B chromosomes in *C. modestus* and *S. insculpta*, according Martins et al. (1996). The first one suggests that these chromosomes arose in some ancestor of the family and were eliminated in species where they are not found today. The second one suggests that B chromosomes have a more recent and independent origin in the species that bear it.

The results obtained in this study provides more information about the occurrence of B microchromosomes in the curimatids, confirming its presence in *Cyphocharax spilotus, C. modestus* and *Steindachnerina insculpta*, previously described in other populations, and showing new events in *Cyphocharax voga, C. saladensis* and *Steindachnerina biornata.* These data confirm the outstanding characteristic of this type of chromosome in this group of fish and its mitotic and meiotic instability and allow a further discussion about the origin of Bs in the family Curimatidae.

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RESEARCH ARTICLE

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Cytogenetics of a parthenogenetic Arctic species of *Micropsectra* (Diptera, Chironomidae)

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Abstract

Micropsectra sedna (Oliver, 1976) is a parthenogenetic midge from the Canadian Arctic. The parthenogenetic mechanism is apomictic thelytoky, with a restitutional division during oogenesis, as found in other parthenogenetic Chironomidae. It is triploid, with two similar chromosome sets, and the third is relatively dissimilar, pairing little with the diploid set. Two karyotypes were observed: a single individual with eight polytene elements in the salivary glands (3n=12), considered standard, while the majority of larvae showed only seven polytene chromosomes (3n=11). Hybrid speciation is considered likely, although chromosomal recombination following the origin of thelytoky has played some part in karyotype evolution. A single morphologically distinct larva was also found, which might be the donor of the haploid chromosome set. The apomictic restitutional system is compared to that of the other, independently derived, parthenogenetic Chironomids to assess the extent of similarity between species.

Keywords

Chironomidae, parthenogenesis, polytene chromosomes, hybridization, chromosome recombination

Introduction

Parthenogenesis, in the forms of arrhenotoky, deuterotoky, or thelytoky, is a quite common phenomenon in the animal kingdom (Suomalainen 1962). Thelytoky, in which females produce exclusively female progeny in the absence of genetic fertilization, is the most widespread and most mechanistically diverse form of parthenogenesis (Hartl 1971).

Thelytoky itself is present in a wide variety of forms. The mechanism for the maintenance of thelytoky may be automictic, in which at least the first meiotic division is normal, the chromosomes pairing at prophase and forming bivalents. The zygoid phase is restored by the restitution of anaphase I or metaphase II chromosome plates, fusion of second division products or endomitosis in cleavage nuclei. Alternatively the mechanism may be apomictic, in which meiotic features may be partly or wholly absent, the one or two maturation divisions being equational. Thelytoky may be complete, it being the only manner of reproduction; or it may be cyclical, where it alternates regularly, or under the influence of environmental factors, with amphimixis or arrhenotoky. Many thelytokous species are also polyploid, allopolyploidy being more common (Gregory and Mable 2005). These allopolyploids also tend to be of hybrid origin (Bullini 1994), and while it is often assumed that polyploidy and thelytoky arose together, there is no proof of this (Gregory and Mable 2005).

The eggs of many thelytokous forms require penetration by the sperm of the same or related species before they develop, but this has not been found in previously described thelytokous Chironomids (Scholl 1956, 1960; Porter 1971).

This paper will examine the cytology of *Micropsectra sedna*, a member of the chironomid subfamily Chironominae, and compare it to other independently derived, thelytokous Chironomids of the same subfamily (Porter 1971), or the subfamily Orthocladiinae (Scholl 1956, 1960).

Material and methods

Second and fourth instar larvae of *M. sedna* were collected from Char Lake, Resolute Bay, North West Territory (now Nunavut), Canada (74°42'N; 94°53'W), in May and July 1970, packed at 4°C and air freighted to Melbourne, Australia. The stock was then split, with some set up at 15°C and the rest kept in an environment varying from 0–10°C (ave. 6°C). Only the lower temperature colony bred successfully. It was maintained in rearing units consisting of a 25cm × 25cm × 12.5cm plastic container connected to a constant air supply. The containers were placed in wooden cages with perspex sliding doors, and the sides of the cages were predominantly fine nylon mesh to allow adequate ventilation. The larvae were fed a finely ground mixture of chicken pellets, dog cubes, soya bean flour and 'Pro-vita' wheat hearts, alternating with several drops of a broth culture of the bacterium *Pseudomonas aeruginosa* (Schröter, 1872) (after Wool and Kugler 1968, who used *Escherichia coli* (Escherich, 1884)).

Several adults emerged from the 15°C tank, but only one oviposited and the eggs failed to develop. Between 11 June and 23 July 1970, 48 adults emerged from the refrigerator culture and 33 oviposited. The clutches varied between 96 and 372 eggs, averaging 201, of these four showed absolutely no sign of development. Of the remaining 29 clutches 2144 out of 2655 eggs hatched (81% hatchability).

Late fourth instar larvae, at the stage just after the appearance of the anlagen of the adult eye (phase 7–8 of Wülker and Götz 1968), were used to characterize the

salivary gland chromosomal banding pattern. The glands were dissected from fresh larvae and stained in 1.6% orcein in 80% lactic acid - propionic acid (1:1) (Martin et al. 2006). The cover glasses were ringed with nail varnish, and the slides stored in a deep freeze.

Photographs from fresh 'semipermanent' preparations were used in the construction of chromosome maps. The chromosomes have been numbered 1 to 8 and arbitrarily given left and right ends. The major divisions have been numbered consecutively throughout the karyotype and each of these subdivided into minor divisions, denoted by letters, at readily identifiable bands, trying to limit the number of bands within a minor division to less than 12. The bands within these minor divisions were numbered (although the numbers are not shown in the figures due to lack of space), so any band can be identified by the number of the major division, the letter of the minor division and the number of the band within that minor division, e.g. band 16a9 can immediately be identified as in major division 16 on chromosome 3, and band 9 in minor division a. Where segments of chromosomes were heterozygous for an inversion or a deletion, the 'diploid' sequence has been taken as the standard sequence. The advantages of a consecutive numbering system over a system in which each chromosome is numbered independently have been discussed by Martin (1969).

For the study of oogenesis in the early embryos, whole or partial egg clutches were treated with 2% sodium hypochlorite for 10–20 sec, permitting release of the eggs from the mucopolysaccharide sheath. The eggs were fixed for 10 min in 45% acetic acid on an albuminized slide and squashed with a siliconized cover glass, which was flicked off after freezing with liquid nitrogen. The material was then dehydrated through an ethanol series, post-fixed overnight in Kahle's fixative with water (1 part glacial acetic acid, 6 parts formalin, 15 parts ethanol, 30 parts distilled water), dehydrated, and extracted for 2 days in 1:1 methanol-chloroform mixture. The preparations were Feulgen stained (Darlington and La Cour 1962), and then rinsed for about 30 sec. in slowly running tap water (Demalsy and Callebaut 1967), dehydrated, and mounted in DePeX.

Results

Polytene chromosomes

M. sedna is triploid, based on 3n=12. There are two karyotypes present in the Char Lake collection, one with eight polytene elements in the salivary glands, the other with seven (3n=11). The biotype with eight polytene elements is considered Standard, despite the fact that it was only found once compared with about 20 of the biotype with seven polytene elements, since it has a greater likelihood of being closer to the original karyotype (see below).

The eight chromosomes of the Standard karyotype can be divided into two groups. Chromosomes 1 to 4 (Fig. 1) consist of two homologues, and will be referred to as the diploid set. Chromosomes 5 to 8 (Fig. 2) occur as single entities, and will be termed

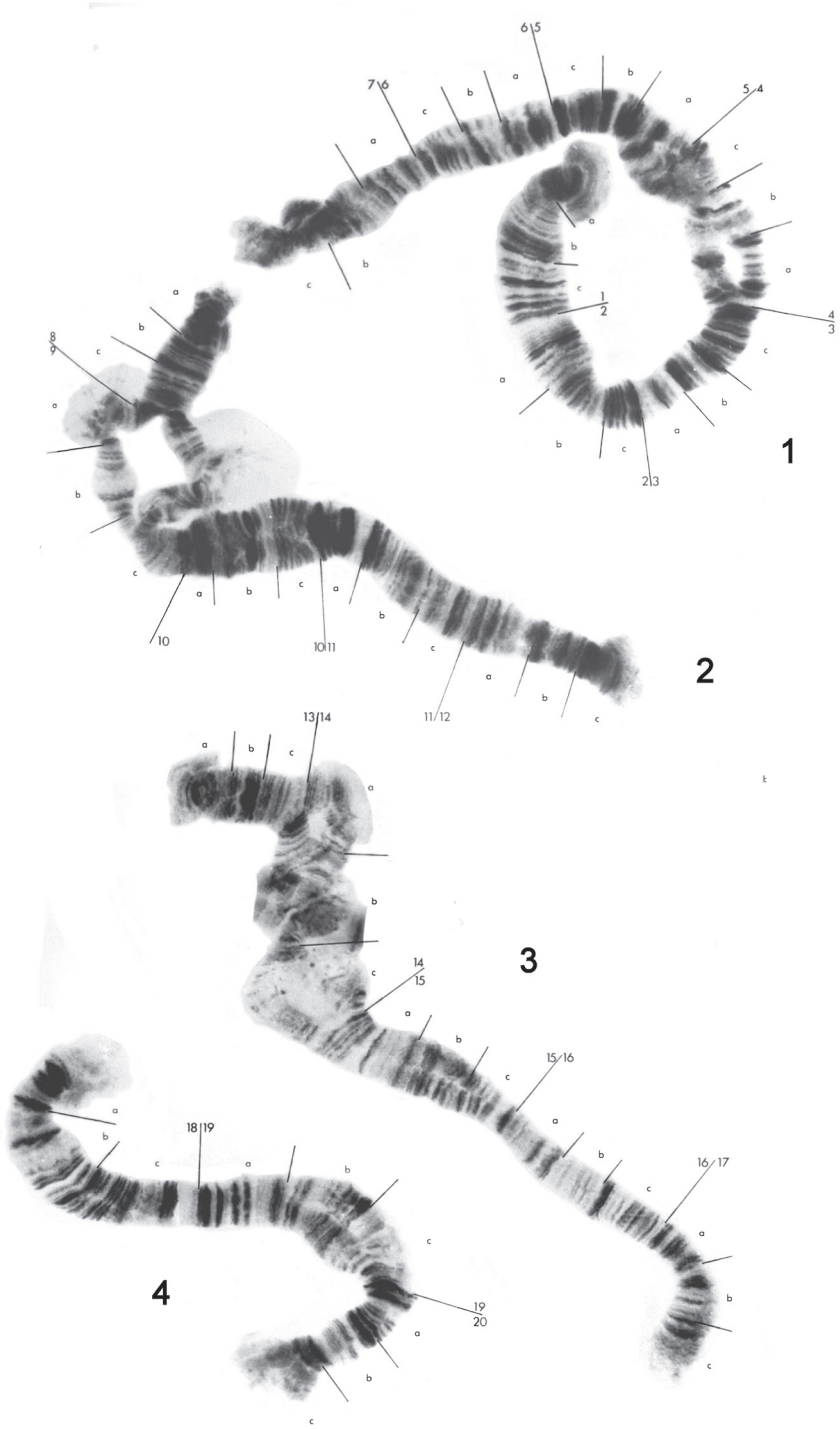


Figure 1. Polytene chromosome maps for chromosomes 1 to 4 of the eight-chromosome specimen of *Micropsectra sedna*.

the haploid set. There is some homology between members of the haploid and diploid sets, however it is impossible to trace the banding pattern of one set completely in the other, and better material would have to be used in order for this to be achieved. Breakdown of pairing is evident in the diploid chromosomes, especially in the vicinity of puffs and bulbs. These do not appear to be due to any structural rearrangements.

Chromosome 1 (Fig. 1) has been divided into seven major divisions. As with the rest of the diploid set, no centromere is obvious. There appears to be some swelling, which could be a poorly developed bulb, in 4c. There are regions of non-pairing in 4a-c, 7b-c and, in some individuals, 6a-b. Readily identifiable regions are the dark sets of bands in 2a and 2c, a dark set of at least three bands at the end of 3c and five dark regions from the middle of 5a to the end of 5c.

Chromosome 2 (Fig. 1) has been divided into five divisions, 8 to 12. There is a puff in 9a and a bulb in 9b. 9a and 9b are normally not paired and the separation may extend to the end of the chromosome at 8a. The best marker areas are a group of distinct pale separated bands in l0c followed by a dark region in 11a and the dark thick band at the start of 12c.

Chromosome 3 (Fig. 1) has been divided into five divisions, 13 to 17. Unfortunately region 14b was not distinct in any preparation There is a large swelling or bulb in 15b. The best marker areas are the dark bands at the end of 13b, the end of 15c, the end of 16a, and the start of 16c.

Chromosome 4 (Fig. 1), the smallest of the diploid set, has been divided into three divisions, 18 to 20. There are no distinct puffs or buIbs, but the ends of this chromosome are characteristically rather diffuse. There is a tendency towards non-pairing at 19b and 19c. Obvious regions are the dark patch in the middle of 18a, five discrete dark bands at the start of 19a, two dark bands at the start of 20a and two dark bands at the start of 20c.

Chromosome 5 (Fig. 2) has been divided into five regions, 21 to 25. It is characterized by a large puff in 23c. Good markers are the dark patches of bands in 21c and 22a, pairs of bands in 22b, 22c and 23a, the dark swollen area 24b and two groups of three dark bands at the start of 25a and 25b.

Chromosome 6 (Fig. 2) has been divided into five regions, 26 to 30. It is characterized by two heterochromatic blocks in 27b, either of which could be taken for a centromere, and a small bulb at 28b. The most obvious markers are the dark region in 28c followed by a pale group of separated bands in 29a, and three dark bands at the end of 30b.

Chromosome 7 (Fig. 2) has been divided into six regions, 31 to 36. It is characterized by a heterochromatic block in 33b, which again is somewhat similar to a centromere, and a distinct constriction at the end of 32c. There are few distinct regions of this chromosome, the most obvious being the doublet at the end of 31c, and the dark patch of about four bands at the start of 35b.

Chromosome 8 (Fig. 2), the shortest of the haploid set, has been divided into four regions, 37 to 40. It has a small puff in 39b, which is quite variable in size, frequently being absent in the biotype with seven polytene elements. The best marker regions are the dark patch of close bands at the end of 37c, five dark separated bands at 38a-38b, the dark bands at the start of 40a and the two dark pairs of bands in 40b.

Figure 2. Polytene chromosome maps for haploid chromosomes 5 to 8 of the eight-chromosome specimen of *Micropsectra sedna*. The small, partially paired segment at region 26 of chromosome 6 is from region 8 of chromosome 2.

Homology between the chromosome sets

It is usually rather difficult to pick homology, because generally the diploid set is not overly stretched and there are regions of non-pairing, whereas the haploid chromosomes are quite stretched.

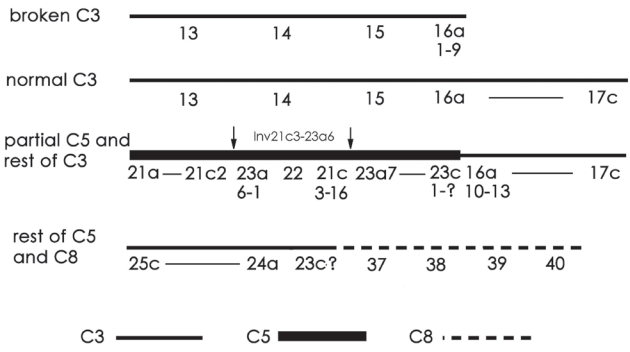
Region 8 in chromosome 2 and region 26 in chromosome 6 appear to be the same because they have been found to pair occasionally, as shown in Fig 2. This is the only homologous pairing seen between members of the haploid and diploid sets. Also the sequence 10a3 to 11a8 in chromosome 2 appears to have been inverted and is present as 29c6 to 28b6 in chromosome 6.

In chromosomes 3 and 5, there is a degree of similarity between 15b17–16b2 and 21b4–22c2, likewise between 13a-c and 25c-a. There also appears to be close correspondence between chromosomes 4 and 8. The sequence from the start of 18a to somewhere in the swollen 19c is probably homologous to the sequence from the start of 37a to the puff in 39b. There are also similarities between 20a8–20b3 and 39b7–39c3, and between the dark bands at the start of 20a and 40a. The end of chromosome 4 is too indistinct to postulate further homology. This leaves chromosome 7 as the possible homologue of chromosome 1, and there are some regions of possible homology, such as 5c-6b with 34a-35a, to support this suggestion.

Chromosomal rearrangements

The biotype with seven polytene elements, which is the most common in the material analysed, differs from the biotype with eight polytene elements by the break up of chromosome 5 and its addition to chromosomes 3 and 8.

There appears to have been a break in one of the homologues of chromosome 3, between the two dark groups of bands in 16a just after 16a9. A second break occurred in chromosome 5 just before the puff in 23c. In addition, chromosome 5 has an inversion of about 21c3 to 23a6 (Inv21c3–23a6), the breakpoints of which are indicated by arrows below. The region 21a to 23c, carrying the inverted sequence, is then joined to one of the chromosome 3 homologues at 16a10 (Fig. 3a) to give the following complex:



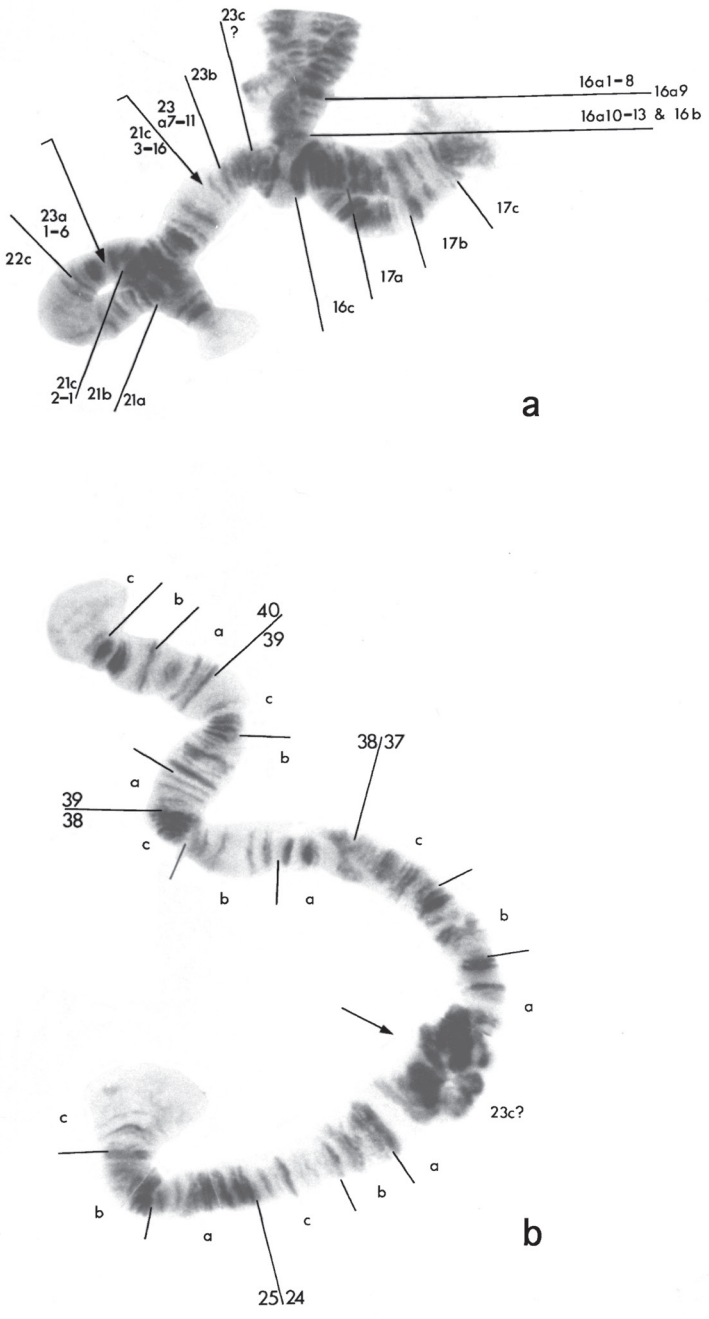


Figure 3. Modified chromosomes of the seven-chromosome biotype of *Micropsectra sedna*. **a** Part of chromosome 5, regions 21a to 23c7, attached to chromosome 3. Arrows show the inversion in the chromosome 5 segment. C3 – chromosome 3; C5 – chromosome 5 **b** Diagrammatic representation of chromosomes in **a c** Other part of chromosome 5 attached to chromosome 8. Arrow indicates the point of fusion.

The reciprocal product is a deleted chromosome 3, comprising regions 13 to 16a9. An unresolved question is whether this deleted chromosome has a telomere at 16a9 and, if so, where it came from. The other chromosome 3 homologue remains unaltered. The rest of chromosome 5 is joined at its proximal end (23c), to region 37 at the left end of chromosome 8 (above and Fig. 3b).

Maturation divisions

At early anaphase I, after 60 min, the chromosomes are pale staining and quite despirallized (Fig. 4a). By the end of anaphase, after 90 min, they are very condensed and stain strongly (Fig. 4b). At least 7 chromosomes are present at some poles although 6 or less than 6 are usually seen. After 120 min very despirallized, disorganized stages are present (Fig. 4c); these probably correspond to the restitution stage reported in *Paratanytarsus grimmi* (Schneider, 1885) (formerly *Lundstroemia parthenogenetica* Freeman, 1962) (Porter 1971). No second anaphase was seen.

Up to what is regarded as the restitution stage, the maturation mechanism appears to be essentially the same as *P. grimmi* (Porter 1971), differing only in the degree of chromosomal contraction, although this may itself be an artefact.

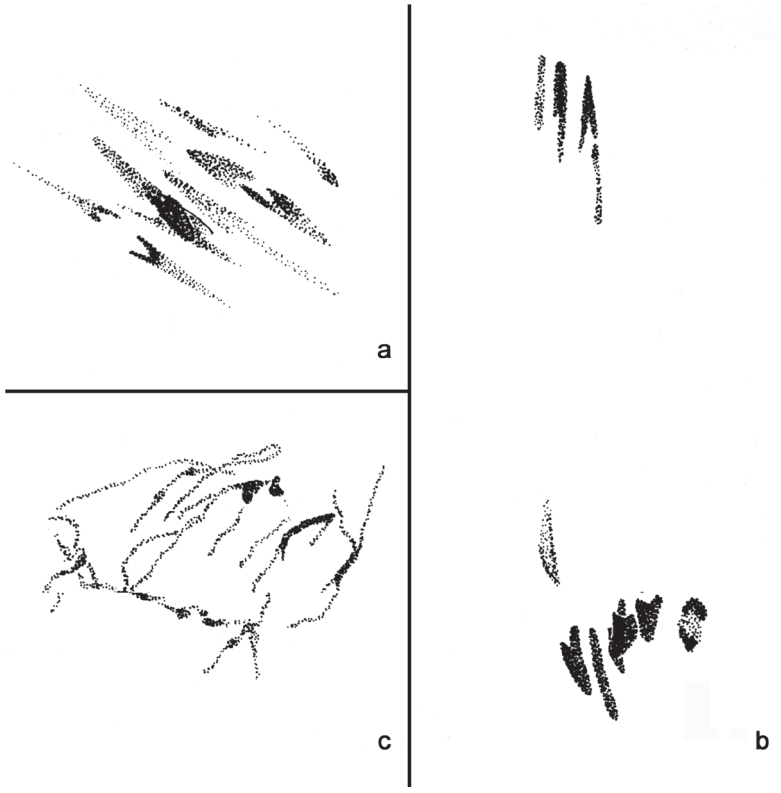


Figure 4. Maturation divisions: **a** Early anaphase I **b** Late anaphase I **c** "Restitutional" stage.

Occurrence of a diploid form

One larva out of the 31 analysed was found to be diploid, 2n=8, rather than triploid. This diploid larva was in extremely poor condition and it could not be sexed. It also was not possible to obtain publishable photographs of the polytene chromosomes or to compare the banding pattern with those of the triploids.

There are four polytene chromosomes, showing considerable non-pairing of homologs; however there does not appear to be any inversion heterozygosity. Three chromosomes are quite long, two of them having heterochromatic blocks which may be centromeres. The small fourth chromosome has a number of puffs and is unpaired for most of its length.

The larval morphology of the diploid differed somewhat from the triploids, although still *Micropsectra*. The size and shape of the labial plates and mandibles of the two types were found to be similar, however the antennae and the length of the setae on the ultimate abdominal segment were markedly different.

Discussion

Origin of the diploid and haploid chromosome sets of *M. sedna*

As indicated above, the presumed standard polytene chromosome complement of this species comprises four diploid chromosomes and four haploid chromosomes, with relatively different banding sequences. As well, two of the haploid set have a large heterochromatic block, which is postulated to be the centromere, while there is no development of heterochromatin to indicate the centromere locations in the diploid set. Despite these differences and the very limited pairing observed between members of the two sets, there appears to be some relationship between each of the chromosomes of the haploid set and a chromosome of the diploid set: C1 with C7, C2 with C6, C3 with C5, and C4 with C8. This suggests that *M. sedna* is a triploid of hybrid origin, with a diploid chromosome set (C1-C4) from one parent, and a haploid set (C5-C8) from the other parent. The extent of difference between the two sets suggests that the two species may not have been particularly closely related. One possibility is that the single diploid larva in our sample contributed the haploid set of *M. sedna,* but the poor condition of the chromosomes of the specimen make this only speculation, but supported by the presence of heterochromatic blocks in two chromosomes, as seen in the haploid set of *M. sedna*. The morphological differences of the diploid larva are not of such a nature as to immediately rule out the possibility of viable hybridization. *Micropsectra* is one of the most species rich genera in the Chironomidae (Ekrem et al. 2010) with more than 130 species in the Holarctic region. However, only a few have been studied cytologically (Michailova 1989), and these are not closely related to *M. sedna*. So, while there may be potential for hydridization in a speciose genus, we cannot suggest what the parental species of *M. sedna* might be.

It is also possible that the differences between the two chromosome sets are due to extensive recombination and mutation of the chromosomes subsequent to the development of thelytoky. There are some factors of the arctic environment that have been suggested as explaining why triploid thelytoky and selection, including chromosomal recombination and mutation may be advantageous. Most of these were outlined by Downes (1962), and relate to the arctic as a marginal and variable environment for insects. Downes suggested that polyploidy, hybridization and apomixis provided stable genetic variability that permits the insects to successfully exploit this extreme environment. The adoption of thelytoky also permits a species to avoid another of the problems of this extreme environment - the limited time when temperatures are high enough to permit flight, and the strong winds that limit the ability to swarm and hence find partners. Some species overcome this problem by mating on the ground, but this still requires males, which are more susceptible to cold than are females. Oliver and Danks (1972) found that arctic species of Tanytarsini tend to have fewer males than females. Adoption of thelytoky can therefore be further advantageous in eliminating the need for these more susceptible males.

While Downes (1962) saw the absence of meiosis as leading to a stable genotype, this need not necessarily be true. Polyploidy provides genetic variability on which selection can act, perhaps to adapt the triploid to a niche with less competition from the parental forms. The high levels of chromosomal rearrangement in *M. sedna* could also have selective advantage if they have occurred subsequent to the origin of the polyploidy, even if no hybridization was involved. Chromosomal recombination can occur freely in the absence of meiosis and potential phenotypic variability could occur by bringing different genes into close proximity and, as recent genomic studies suggest, inactivating existing genes or creating new ones at the break points (Furuta et al. 2011). Clones with advantageous mutations would become more prevalent than those with less advantageous genomes. This may well be the explanation for the greater prevalence of the seven-chromosome form of *M. sedna* compared to the Standard eight-chromosome form.

The restitutional mechanism

Where the mechanism of thelytoky is apomictic, restitution is one possible means of restoring the chromosome number. Restitution is the annulment of a maturation division by the immediate reunion of the separating elements, and although rather widespread in occurrence is still quite a rare phenomenon. It appears to be the normal mechanism in parthenogenetic Chironomids, as it has been recorded in the Orthocladiine parthenogens examined by Scholl (1956, 1960) and in the Tanytarsine *P. grimmi* (Porter 1971), as well as in the present species. After prophase the univalents assemble at the spindle equator, and undergo an abortive first division, followed by the formation of a restitutional spindle on which an apparently normal mitotic division takes place. The resulting nuclei each take part in segmentation.

There are certain specific differences relating to the arrangement of the chromosomes in prophase, the degree and pattern of despirallization during the abortive division, and the extent of development of the second division equatorial plate, but the mechanism is identical in all of them. Scholl (1960) indicated that orientation of the centromere region is random in the abortive first division of the Orthocladiines because the univalents separated to the poles at anaphase in quite a variable manner, while Porter (1971) found that variation in *P. grimmi* was rare, perhaps 1 in 20. Scholl observed some terminal associations during prophase of the Orthocladiines, which were not seen in *P. grimmi*, and it is possible that these persist to metaphase and have some effect on the orientation of the univalents at metaphase I. There were insufficient anaphases to make any comment about *M. sedna* in this regard.

Since thelytoky in these diverse Chironomids must have arisen independently, why have they all followed the same mechanism? It might be suggested that the ability to develop without the incorporation of the male genome is a rather common occurrence, but perhaps latent and not manifested until triggered by hybridization, environmental stimulus or mutation. Whatever the stimulus, the maturation will then proceed in a genetically determined manner. An important component of this genetic determination may be the ability of the centrosome to self-reassemble in the divisions of parthenogenetic insects (Yang et al. 2008). Beyond this, it may also be logical to assume that the genetic resemblance that places organisms in the same taxonomic grouping, may lead to a similar mechanism of thelytoky being more likely to become established - in this case, apomixis with a restitutional division.

This does not mean that the mechanisms will be identical in all details, as noted above. The significance of the differences in the extent of despirallization during the restitution division is unknown. Scholl's (1960) figures indicate that the chromosomes of *Limnophyes virgo* Remmert, 1953, more so than the other species he studied, undergo considerable extension during anaphase I, particularly near the centromere region. *M. sedna* also shows this peculiarity, far more so than *P. grimmi* (Fig. 4, c.f. Fig. 2, Porter 1971). However, in this case it is possible that the despiralization observed in *M. sedna* may be an artefact of the laboratory rearing conditions. As an arctic species it would rarely experience temperatures above about 2–3°C in nature, whereas the eggs were kept at about 5–6°C in the laboratory and the despiralization may have been a consequence of this.

The parthenogenetic system of *M. sedna* therefore more closely resembles that of the other known Tanytarsine parthenogen *P. grimmi*, in being triploid, with chromosomal polymorphism of probable hybrid origin. The parthenogenetic species in the relatively distant Tribe Orthocladiinae, studied by Scholl (1956, 1960), are more variable in that only some are triploid, some have additional germ-line limited (K-) chromosomes, and extent of polymorphism varies from a single heterozygous inversion to complex intrachromosomal rearrangement. *M. sedna* does seem to share chromosomal despirallization during the restitution division with *L. virgo*, more so than with the more closely related *P. grimmi*, but this feature seems to be a sporadic species-specific character.

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RESEARCH ARTICLE

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Heterochromatin heterogeneity in *Hypostomus* prope *unae* (Steindachner, 1878) (Siluriformes, Loricariidae) from Northeastern Brazil

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Abstract

Cytogenetic analyses using C-banding and chromosomal digestion by several restriction enzymes were carried out in four populations (named A, B, C and D) of *Hypostomus* prope *unae* (Loricariidae, Hypostominae) from Contas river basin, northeastern Brazil. These populations share 2n=76 and single NORs on the second metacentric pair but exclusive karyotype forms for each locality. Populations A and B presented conspicuous terminal and interstitial heterochromatic blocks on most of acrocentric chromosomes and equivalent to NORs with differences in both position and bearing pair. Population D showed evident marks at interstitial regions and interspersed with nucleolar region while population C presented interstitial and terminal heterochromatin segments, non-coincident with NORs. The banding pattern after digestion with the endonucleases *Alu* I, *Bam* HI, *Hae* III and *Dde* I revealed a remarkable heterogeneity within heterochromatin, allowing the identification of distinctive clusters of repeated DNA in the studied populations, besides specific patterns along euchromatic regions. The analysis using restriction enzymes has proved to be highly informative, characterizing population differences and peculiarities in the genome organization of *H.* prope *unae*.

Keywords

C-banding, heterochromatin, ichthyofauna, restriction enzymes

Introduction

Restriction enzymes (RE) represent a powerful tool for studies about DNA organization (Lima-de-Faria et al. 1980). Such bacterial endonucleases recognize and cleavage target-sequences in the double-strand DNA, providing a highly specific pattern of chromosomal banding according to each enzyme (Lloyd and Thorgaard 1988). The removal of DNA fragments allows studying both structure and base composition of specific chromosomal regions (Lorite et al. 1999; Sanchez et al. 1990, 1991; Lozano et al. 1991; Bianchi et al. 1985). Therefore, the RE banding pattern is an exceptionally sensitive method in heterochromatin analysis (Pieczarka et al. 1996), being able to reveal a higher degree of heterogeneity and more refine comparative analyses than the traditional C-banding itself.

In spite of the intensive application of restriction enzymes in chromosomal analyses of several animal groups (Miller et al. 1976; Kaelbling et al. 1984; Lima-de-Faria et al. 1980; Bianchi et al. 1985; Marchi and Mezzanotte 1988, 1990; Juan et al. 1990; Pieczarka et al. 1996), a few studies of RE-based heterochromatin differentiation are reported in fish chromosomes, being restricted to some groups such as Characidae (Kantek et al. 2007; Maistro et al. 1999), Prochilodontidae (Maistro et al. 2000), Pimelodidae (Swarça et al. 2005; Carvalho and Dias 2005), Salmonidae (Lloyd and Thorgaard 1988; Sanchez et al. 1990, 1991; Lozano et al. 1991; Albuín et al. 1994), Muraenidae (Cau et al. 1988) and Scophthalmidae (Bouza et al. 1994).

Within the genus *Hypostomus* Lacépède,1803, heterochromatin can be associated to heteromorphic chromosomes (Cereali et al. 2008; Kavalco et al. 2004, 2005), sex chromosomes (Artoni et al. 1998) and polymorphism cases (Rubert et al. 2008). In addition, species of this genus usually present a remarkable variability in both distribution and composition of heterochromatin (Artoni and Bertollo 1999). However, these data refer to C-banding and/or fluorochrome staining while studies using enzymatic digestion have not been reported in the genus or the family Loricariidae so far.

The goal of the present work was to analyze comparatively metaphase chromosomes of *Hypostomus* prope *unae* (Steindachner, 1878) by C-banding and RE digestion in order to refine previous cytogenetic studies (Bitencourt 2010) among four populations of this species along a poorly studied coastal river basin in northeastern Brazil.

Methods

Forty-six specimens of *Hypostomus* prope *unae* from four collection sites in Contas river basin were analyzed, being 10 (3 males, 2 females and 5 immature) from the main channel of Contas river (13°51'51"S, 40°04'54"W), 10 (6 males, 1 female and 3 immature) from Preto do Costa river (13°45'84"S, 39°56'47"W), 15 (9 males and 6 immature) from Oricó river (14°08'03"S, 39°21'30"W), and 11 (4 males, 4 females and 3 immature) from Preto do Criciúma river (13°55'45"S, 39°57'57"W) (Fig. 1).

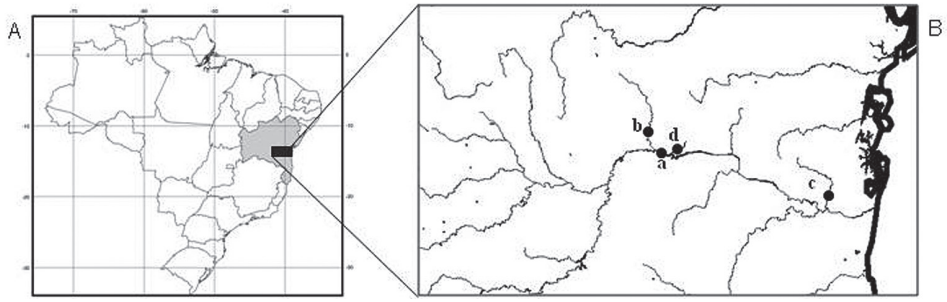

Figure 1. A–B Collection sites **A** Map of Brazil, highlighting the state of Bahia in northeastern region; **B** Contas river basin and respective sampling sites: **a**- Contas river, **b**- Preto do Costa river, **c**- Oricó river, **d**- Preto do Criciúma river.

Voucher specimens were identified by Dr. Claudio Zawadski from Universidade Estadual de Maringá (UEM) and deposited in the fish collection at NUPELIA – UEM, Maringá, PR, Brazil (NUP 9811, 9814). These four populations are referred as A, B, C and D, respectively.

Metaphase chromosomes were obtained from kidney cells as described by Bertollo et al. (1978) after mitotic stimulation using yeast suspension (Lee and Elder 1980) or, alternatively, Munolan® (bacterial and fungal antigens) diluted in water (1 pill per 0.5mL of water), as suggested by Molina (2001). The chromosomes were classified into metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a), as commonly described in fish (Levan et al. 1964). The fundamental number (FN) was established taking into account that m, sm and st chromosomes are bi-armed while chromosomes bear one chromosomal arm.

C-positive heterochromatin was detected according to Sumner (1972), with slight modifications. *In situ* digestion using restriction enzymes was performed as proposed by Mezzanotte et al. (1983), with modifications. Concentration and incubation tests were extensively performed to optimize the results. After defining the best concentration (Table 1), we added 30 µl of each enzyme solution (diluted in specific buffer and distilled water) onto chromosomal preparations. The slides were incubated in moist chamber at 37°C for specific periods according to each enzyme (Table 1). Afterwards, the slides were washed in distilled water and stained with 5% Giemsa in phosphate buffer (pH 6.8) for 8 minutes.

Results

The specimens from all analyzed populations presented a modal chromosomal number of 2n=76 and distinct karyotype formulae, as follows: 12m+16sm+48st/a (FN= 104) for specimens from population A, 12m+20sm+44st/a (FN=108) for specimens from

Table 1. List of restriction endonucleases (RE) used on the chromosomal preparations of *Hypostomus* prope *unae,* with their respective restriction sites and optimum concentrations and incubation periods obtained in the present work.

Endonucleases	Restriction site	Concentration	Incubation
Alu I	(5'- AG ↓ CT - 3')	0.4 U/ µl	4h
Bam HI	(5'- G ↓ GATCC - 3')	0.5 U/ µl	15h
Hae III	(5'-GG ↓ CC - 3')	0.6 U/ µl	14h
Dde I	(5'- C ↓ TNAG – 3')	2 U/ µl	4h

population B, 10m+14sm+52st/a (FN=100) for individuals from population C and 10m+20sm+46st/a (FN= 106) for those from population D. Furthermore, distinctive patterns of heterochromatin distribution were detected by C-banding. Although populations A and B bear conspicuous terminal and interstitial marks in 17 chromosomal pairs as well as centromeric and NOR-associated heterochromatin, they differ in relation to C-bands position or bearing pair (Figs 2, 3).

Heteromorphic blocks were also evident in both populations. Besides the NOR-bearing pair, 18th, 21st and 37th pairs in population A and the 22nd pair in population B size differences between homologous (Figs 2, 3). Population C was characterized by interstitial and terminal marks in six chromosomal pairs, non-coincident with NORs (Fig. 4). On the other hand, population D presented eight pairs, most of them acrocentric, bearing interstitial C-bands and also interspersed with NORs (Fig. 5).

The digestion pattern using RE allowed identifying inter-population differences in several chromosomal regions but most in heterochromatin as shown in Table 2, where + stands for digested C-band and – stands for undigested heterochromatic region.

Five heterochromatin (or repeated DNA) groups were identified in population A: (a) the heterochromatin from pairs 2, 17, 21, 30, centromeric heterochromatin of pairs 1, 3, and 35, and terminal regions of the 29th pair were digested by all tested enzymes; (b) the chromosomal pairs 16, 18 and 25 lacked any target sequences; (c) pairs 7, 22, 23, 32 and the terminal heterochromatin of pairs 1, 3 and 35 were digested by *Alu* I, *Bam* HI and *Dde* I; (d) pair 28 and the upper portion of the heterochromatic block in pair 29 were digested by *Hae* III, *Bam* HI and *Dde* I; and (e) the 37th pair was digested by *Alu* I (Fig. 2).

In population B, the heterochromatin was divided into six groups: (a) the heterochromatin from pairs 2, 5, 10, 11, 16, 17, 28, 29 and 34 were digested by all enzymes; (b) the chromosomal pairs 18, 22 and 30 lacked the target sequences; (c) the pairs 25 and 36 were digested by *Alu* I, *Bam* HI and *Hae* III; (d) the heterochromatin from pair 8 was digested by *Hae* III and *Dde* I; (e) the 32nd pair was digested by *Alu* I and *Dde* I; (f) and the 21st pair was digested by *Alu* I, *Hae* III and *Dde* I (Fig. 3).

Enzymatic digestion of heterochromatic regions in population C revealed four heterochromatin groups: (a) centromeric region of pair 21 and the terminal blocks in pair 23 remained intact; (b) pair 8 was digested by *Hae* III; (c) pair 15, central portion of heterochromatic block in pairs 23 and were digested by *Bam* HI; (d) pair 19 and the terminal region of pair 21 were digested by *Bam* HI and *Dde* I (Fig. 4).

Table 2. Heterochromatin digestion pattern using the restriction enzymes *Alu* I, *Hae* III, *Dde* I and *Bam* HI per population of *H.* prope *unae*: (+) digested heterochromatin; (-) undigested heterochromatin; (±) partially digested heterochromatin.

Population	C-banded pair.	Restriction Enzyme			
		***Alu* I**	**III*Hae***	**I*Dde***	**HI*Bam***
A	1	+	±	+	+
	2	+	+	+	+
	3	+	±	+	+
	7	+	–	+	+
	16	–	–	–	–
	17	+	+	+	+
	18	–	–	–	–
	21	+	+	+	+
	22	+	–	+	+
	23	+	–	+	+
	25	–	–	–	–
	28	–	+	+	+
	29	±	+	+	+
	30	+	+	+	+
	32	+	–	+	+
	35	+	±	+	+
	37	+	–	–	–
B	2	+	+	+	+
	5	+	+	+	+
	8	–	+	+	–
	10	+	+	+	+
	11	+	+	+	+
	16	+	+	+	+
	17	+	+	+	+
	18	–	–	–	–
	21	+	+	+	–
	22	–	–	–	–
	25	+	+	–	+
	28	+	+	+	+
	29	+	+	+	+
	30	–	–	–	–
	32	+	–	+	–
	34	+	+	+	+
	36	+	+	–	+
C	8	–	+	–	–
	15	–	–	–	+
	19	–	–	+	+
	21	–	–	±	±
	23	–	–	–	±
	26	–	–	–	+

Population	C-banded pair.	Restriction Enzyme			
		***Alu* I**	**III*Hae***	**I*Dde***	**HI*Bam***
D	2	+	+	+	+
	4	–	–	–	–
	18	–	–	–	–
	22	–	–	–	–
	24	–	–	–	–
	27	–	–	–	–
	29	–	–	–	+
	33	–	+	+	–

Heterochromatin regions in population D were also divided into four groups: (a) pair 2 was digested by all enzymes; (b) pairs 4, 18, 22, 24 and 27 were not digested by the tested enzymes; (c) the 29th pair presented target sequences for *Bam* HI; (d) and the 33rd pair was digested by *Hae* III and *Dde* I (Fig. 5).

Independently on the population, the nucleolus organizer regions (2nd pair) were digested by all restriction enzymes, including those samples in which NOR-associated heterochromatin was not detected by C-banding.

In relation to the digestion pattern in euchromatic regions, some conspicuous bands were observed, being specific for each population and enzyme. In general, population A presented a high number of chromosomes bearing *Hae* III bands, whereas populations B and C presented larger amounts of *Alu* I bands. On the other hand, population D was characterized by a large number of chromosomes bearing bands after treatments with all enzymes (data not shown).

Discussion

Chromosomal digestion by restriction endonucleases results in a faint chromosomal staining and identification of a characteristic band pattern according to each enzyme (Lima-de-Faria et al. 1980). The decreased chromatin staining is considered a reliable evidence of the removal of DNA fragments by RE once Giemsa attaches to DNA directly (Miller et al. 1983; Bianchi et al. 1985; Kaelbling et al. 1984), but other factors might also play an important role in this pattern.

A hindered access to chromosomal DNA has been pointed out as an alternative explanation for the banding profiles after RE digestion in some cases (Gosálvez et al. 1986; Marchi and Mezzanotte 1990). Burkholder and Weaver (1977), analyzing the interactions between DNA and proteins in the condensed chromatin of rats and humans, observed a differential sensitivity to enzymatic digestion in some chromosomal regions according to differences in the DNA-attached proteins once they would protect them from enzymatic digestion. However, the relationship of this interaction to chromosomal banding differentiation has not been fully understood yet. In addition,

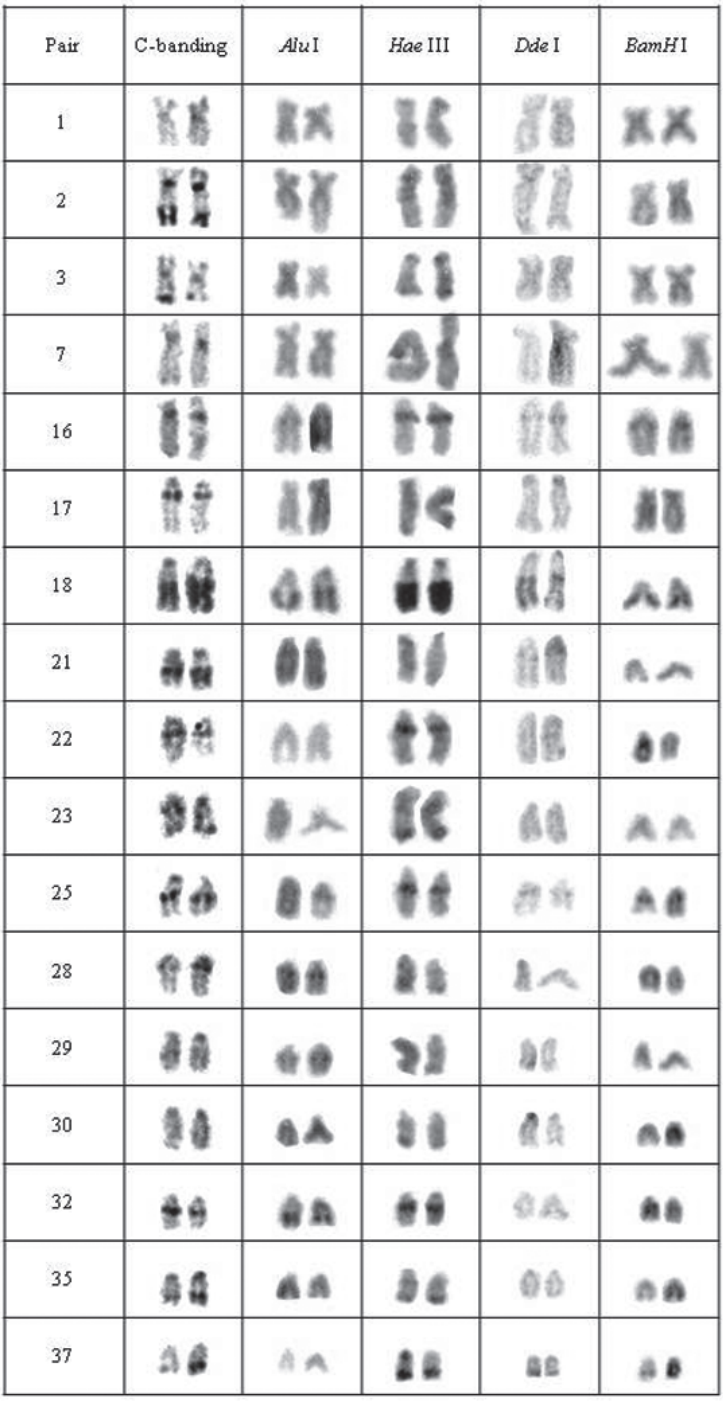

Figure 2. Chromosomal pairs from population A of *Hypostomus* prope *unae* showing the C-positive heterochromatin and banding pattern after digestion with the restriction endonucleases: *Alu* I, *Hae* III, *Dde* I and *Bam* HI.

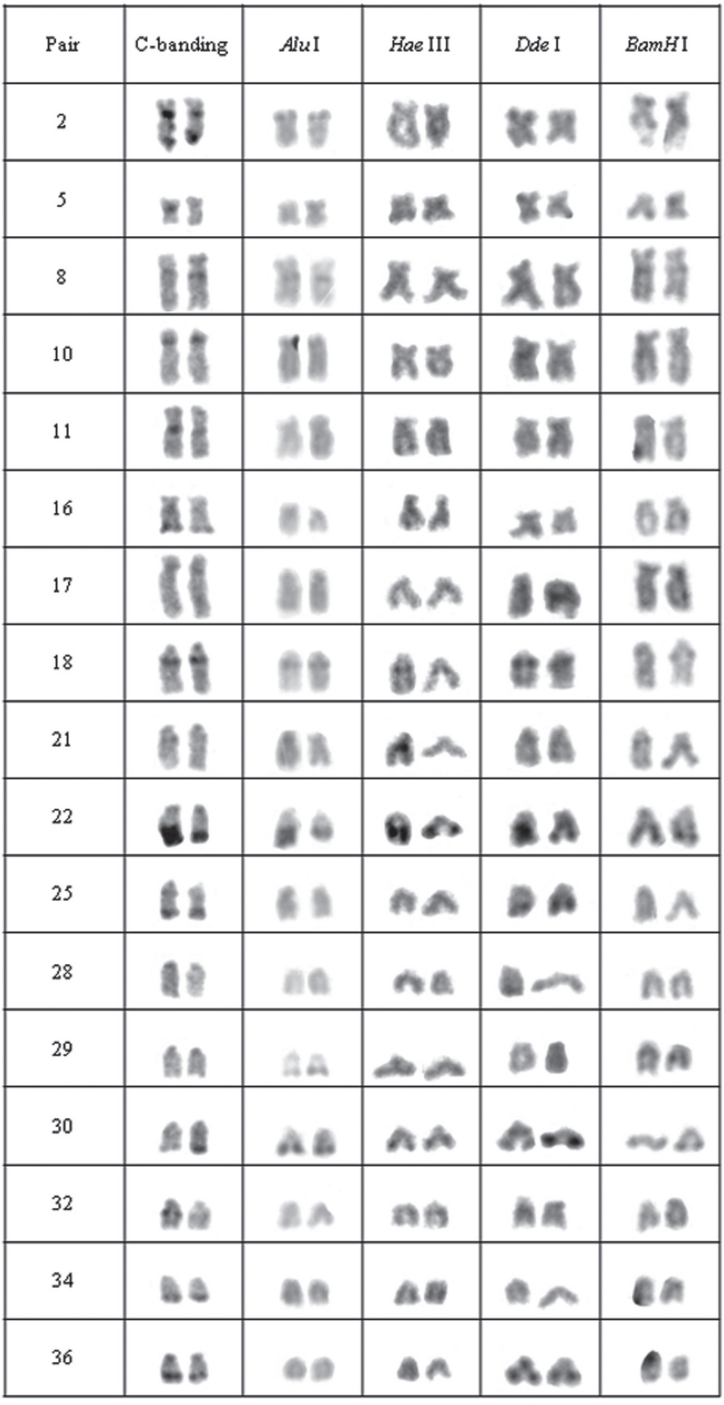

Figure 3. Chromosomal pairs from population B of *Hypostomus* prope *unae* showing the C-positive heterochromatin and banding pattern after digestion with the restriction endonucleases: *Alu* I, *Hae* III, *Dde* I and *Bam* HI.

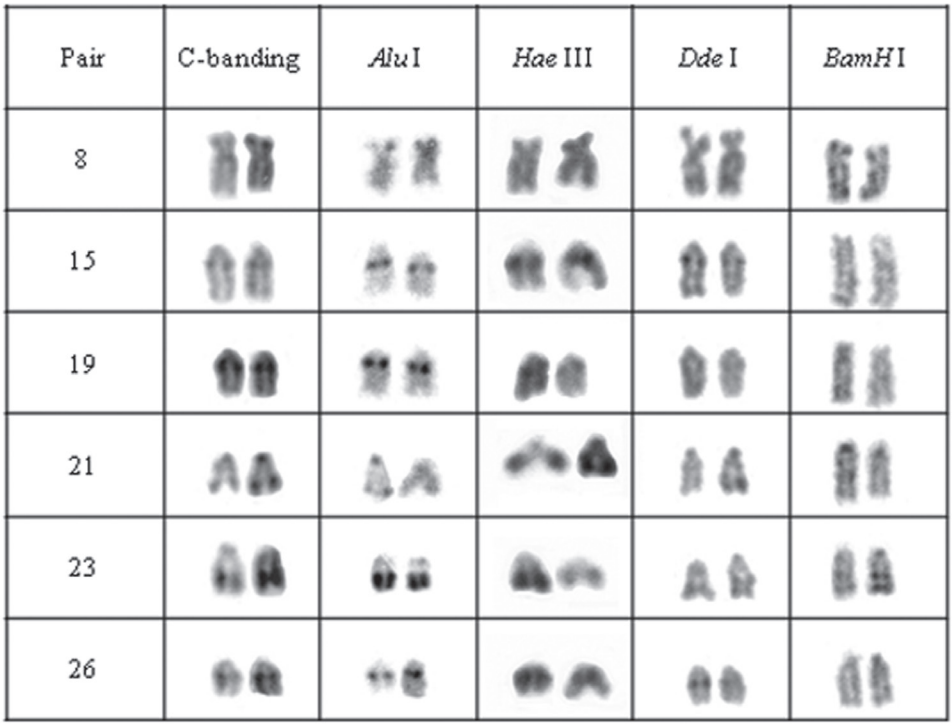

Figure 4. Chromosomal pairs from population C of *Hypostomus* prope *unae* showing the C-positive heterochromatin and banding pattern after digestion with the restriction endonucleases: *Alu* I, *Hae* III, *Dde* I and *Bam* HI.

conformational changes in chromosomal structure putatively account for RE digestion patterns in human chromosomes for instance (Mezzanotte et al. 1985).

In the present work, the application of endonuclease treatments revealed a remarkable heterogeneity within heterochromatin among populations of *Hypostomus* prope *unae*, comprising either distinct or similar chromosomes, and even between heterochromatic segments. Based on these results, it was possible to identify inter- and intra-population (dis)similarities (Fig. 6). Most likely, the tested enzymes cleaved and removed DNA from both euchromatin and heterochromatin as demonstrated by some less stained chromosomal regions. Therefore, the observed bands can be regarded as non-removed DNA portions lacking the RE target sequences.

The present data indicate that some heterochromatin regions in different chromosomes and/or populations share a similar composition, while others would present a unique composition. Thus, the banding pattern observed reflects directly the molecular nature of heterochromatin regions (Sanchez et al. 1991), although a differential access to target sequences by the RE might be present as well.

Such remarkable heterogeneous banding pattern shows that the populations of *H.* prope *unae* bear several heterochromatin families composed of distinct specific types

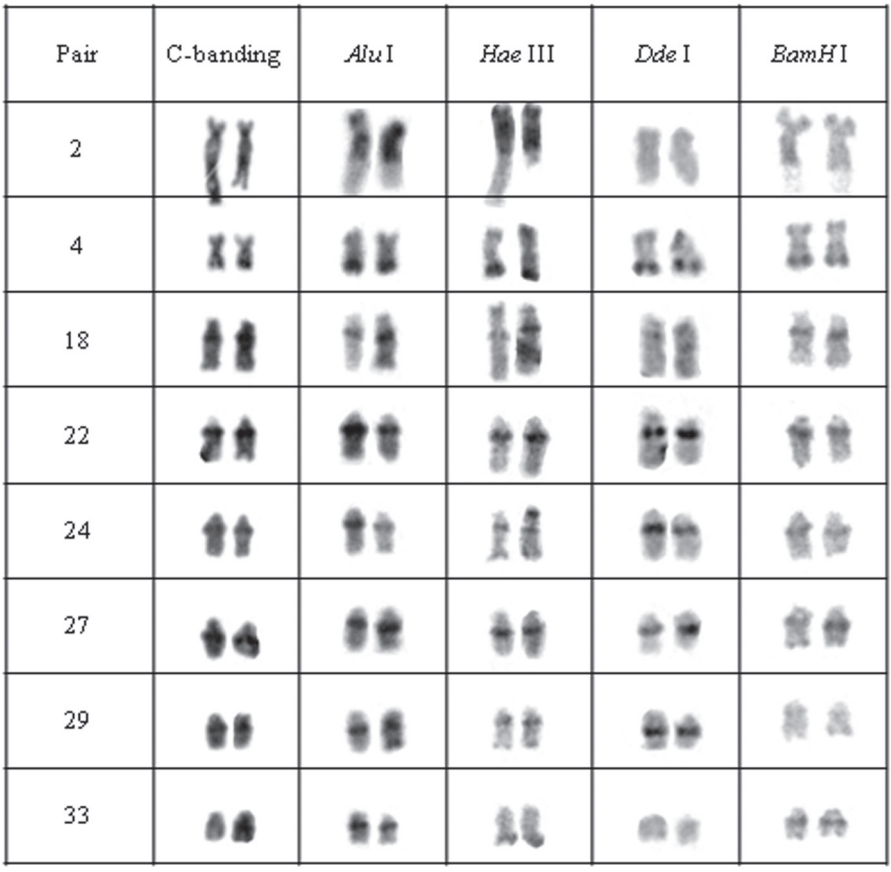

Figure 5. Chromosomal pairs from population D of *Hypostomus* prope *unae* showing the C-positive heterochromatin and banding pattern after digestion with the restriction endonucleases: *Alu* I, *Hae* III, *Dde* I and *Bam* HI.

of highly repetitive DNA. A similar finding was reported in the salmonids *Salmo salar* Linnaeus, 1758 (Albuín et al. 1994) and *Salmo trutta* Linnaeus, 1758 (Sanchez et al. 1991), in which RE digestion resulted in differential heterochromatin digestion in specific chromosomal regions.

According to Schweizer and Loidl (1987), a non-random arrangement of chromosomes during interphase might favor the linkage between certain chromosomal regions and further heterochromatin dispersal to equilocal sites from one chromosome to another, as previously proposed for the distribution of interstitial heterochromatin in other *Hypostomus* species (Artoni and Bertollo 1999). It seems plausible to infer that those heterochromatin segments sharing a similar composition would have a common origin and have been dispersed to similar chromosomal regions of *H.* prope *unae*. Through their karyoevolutionary history, these segments could have been amplified

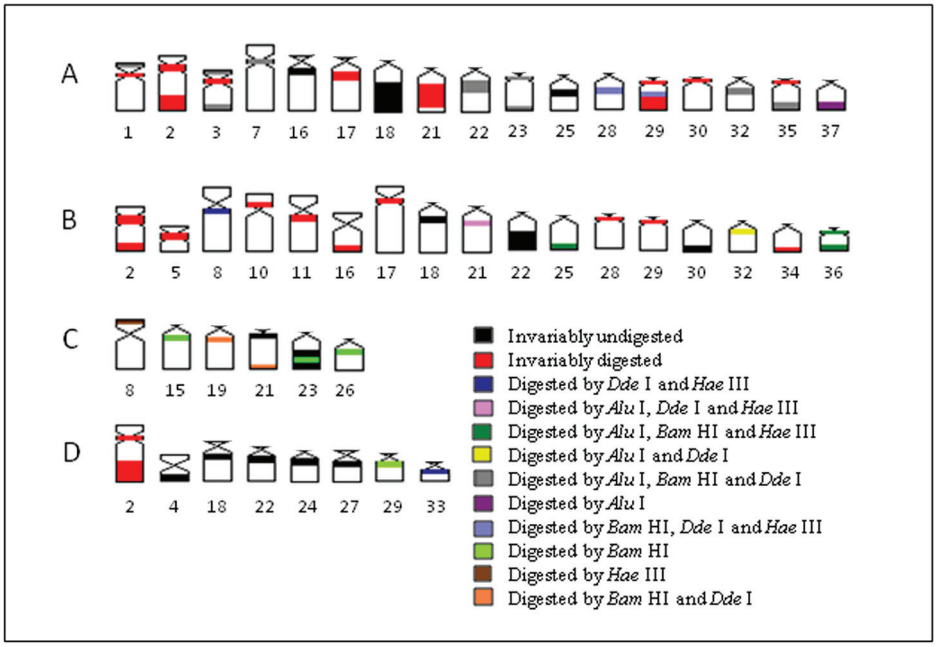

Figure 6. A–D Schematic ideogram of chromosomal pairs from populations **A, B, C** and **D** of *Hypostomus* prope *unae*, showing the combined banding pattern after digestion using *Alu* I, *Bam* HI, *Hae* III and *Dde* I.

or accumulated by unequal exchanges, transpositions and/or regional duplications as similarly hypothesized for the marine fish *Centropyge aurantonotus* Burgess, 1974 (Affonso and Galetti Jr. 2005). Consequently, the chromosomal divergence among the studied populations have possibly been related to rearrangements in the heterochromatin organization and fixed either by genetic drift or by natural selection if some adaptive role is assumed.

Although inter-population differences were detected by both C-banding and RE digestion, some heterochromatin regions remained resistant to enzymatic digestion among populations, mainly in population D, revealing a higher differentiation in the DNA composition and/or heterochromatin organization in the latter. This population is also more divergent than the others because of its high frequency of interstitial C bands instead of terminal ones (Figs 5, 6).

Differences in heterochromatin patterns have been commonly reported in Neotropical fishes, including species from northeastern coastal basins (Jacobina et al. 2009). However, evolutionary mechanisms of heterochromatin differentiation among fish populations are usually related to polymorphic conditions being rarely detected within a single basin (Molina et al. 2008). Thus, the present results indicate that gene flow among *H.* prope *unae* along Contas river basin is absent, favoring the fixation of divergent heterochromatin patterns.

It should be pointed out that the nucleolar organizer regions (2nd pair) was digested by all tested enzymes independently on the population, demonstrating that the distinct target sequences are "concertedly" interspersed along this region, even when NOR-associated heterochromatin was not detected, as observed in population C. Such behavior differs from the pattern observed by Sanches et al. (1990) that reported a differential NOR digestion indicative of a high amount of target sequences for *Dde* I and *Hae* III but a moderate number of restriction sites for *Alu* I.

Moreover, heteromorphic segments were observed between some chromosomal pairs in populations A (pairs 18, 21 and 37) and B (pair 22). Nonetheless, only the 21st pair in population A presented the target sequences for the selected RE, while the other heteromorphic segments proved to be resistant to their digestion activity.

Reports about restriction enzymes in Neotropical fish cytogenetics are scarce what hinders a detailed comparative analysis. However, this approach seems to be highly informative for species characterized by large amounts of heterochromatin as that presently studied, being able to reveal several genomic particularities. Moreover, repetitive DNA sequences might provide efficient chromosomal markers useful for evolutionary studies, identification of chromosomal rearrangements and sex differentiation (Ferreira and Martins 2008).

As commonly reported in fishes of the genus *Hypostomus* (e.g., Milhomem et al. 2010), the present cytogenetic analyses were able to differentiate the four studied populations of *H.* prope *unae*, thereby reinforcing their evolutionary divergence along Contas river basin and their cryptic species diversity.

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RESEARCH ARTICLE

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Karyotyping and *in situ* chromosomal localization of rDNA sites in black cumin *Bunium persicum* (Boiss) B. Fedtsch,1915 (Apiaceae)

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Abstract

The fluorescent *in situ* hybridization (FISH) technique has been applied to somatic chromosomes in the medicinally important species, *Bunium persicum*, to elucidate its karyotypes. The bicolour FISH technique involving 18S-5.8S-26S and 5S ribosomal RNA genes as probes was used to assign physical localization and measurement of rDNA sites on homologous pairs of chromosomes. The two 18S-5.8S-26S rRNA gene sites were at the terminal regions of the short arms of the chromosomes 1 and 2 involving NOR region of chromosome 1. The 5S rDNA sites were found on subtelomeric region of the long arm of the chromosome number 5 and at interstitial regions of the short arm of chromosome 7. Based on direct visual analysis of chromosome length, morphology and position of FISH signals, a pioneer attempt has been made to construct metaphase karyotype in *B. persicum,* an endangered medicinal plant of North Western Himalayas.

Keywords

FISH, karyotypes, *Bunium persicum,* black cumin

Introduction

Black cumin (*Bunium persicum* (Boiss) B. Fedtsch, 2n=14, is a high value medicinal and spice herb that grows as a wild plant in the forests of dry temperate and slopes of high mountainous regions of North Western Himalayas.

In situ hybridisation (FISH) technique has been successful for indentifying chromosome markers and physical mapping in many species of wheat, rice, lentil, and maize. Probes of repeated sequences and multigene families, including rRNA genes have become powerful tools for discerning chromosomal organization also for genetic and taxonomic relationships of agricultural plants (Lapitan et al. 1989; Mukai et al. 1991; Maluszynska and Heslop-Harrison 1991; Tsujimoto and Gill 1991). The nuclear genes encoding both 18S-5.8S-26S (45S) and 5S ribosomal RNA (rDNAs) consist of highly conserved repeat units arranged in one or more tandem arrays up to 10 000bp. In plants, the 18S-5.8S-26S rRNA genes are arrayed within the nucleolar organizing region (NOR), while the 5S rDNA is mapped outside the NOR. The independent localization makes them useful for chromosome identification (Krishnan et al. 2001). The physical mapping of repeated sequences in different crop species has also been widely reported (Irifune et al. 1995; Yamamoto et al. 1999; King et al. 2002; Lavania et al. 2005; Zhang et al. 2005). The high degree of polymorphism detected in their intergenic sequences has been extensively used for studying phylogenetic and genomic relationship among different legume species (Abirached-Darmency et al. 2005; Benabdelmouna et al. 2001). In the present study, FISH technique was applied to wild growing plants of *Bunium persicum* with objectives to elucidate physical localization of repetitive DNA sequences on metaphase chromosomes.

Material and methods

Plant material and preparation of cells for karyotype and FISH analysis

Primary roots from growing plants of *Bunium persicum* in pots at the Department of Crop Improvement, CSK Himachal Pradesh Agricultural University, Palampur, India were excised and pretreated in water for 16 hours at 4°C followed by fixation in ethanol: acetic acid (3:1) mixture for 5 days at room temperature. The root tips were stained in 1% aceto-carmine solution for 15 min and then squashed in 45% acetic acid. Ten well spread metaphase plates with proper chromosome contraction were analysed to prepare the standard karyotype for the species. After removing the cover glass via freezing on dry ice for 15 min, the slides were distained by immersing in 45% acetic acid for 15 min at room temperature. The air dried slides were maintained in a desiccator for at least 24 hours.

Probe labelling

DNA probe for 45S rDNA were generated from the plasmid pTa71 containing 9kb *Eco*R1 fragment of the 18S-5.8S-26S rDNA repeat sequence of *Triticum aestivum* Linnaeus, 1753 (Gerlach and Bedbrook 1979). The 18S-5.8S-26S rDNA was labelled

with biotin-16-dUTP (Roche Diagnostics) by nick translation. The 5S rDNA probe was obtained from onion genomic DNA and was labelled with digoxigenin-11-dUTP (Roche Diagnostics) directly during PCR amplification according to manufacturer's instructions. The probe mixture contained 50% (v/v) deionized formamide, 2XSSC, 10% (w/v) dextransulfate, 5 µg of salmon sperm DNA, 0.1 µg of digoxigenin-labelled 5S rRNA gene probe and 18S-5.8S-26S rRNA gene probe in final volume of 10 µl. This mixture of probes was denatured by putting hybridization mixture in boiling water for 10 min and thereafter kept on ice for 5 min.

In situ hybridization

Chromosomal DNA on the slides was denatured in 70% deionized formamide 10% 20XSSC and 20% DDW at 70° C for 2 min then hydrated in a 70%, 95% and 100% ethanol series at -20° C for 5 min each. Slides were dried immediately with hand blower and kept for 5–10 min at room temperature. 10 µl of probe mixture were applied to each denatured preparation and covered with glass. Slides were then placed in a humid hybridization chamber at 37° C for 15 hours. After hybridization the cover glass was removed by dipping slides in 2XSSC. Slides were then washed in 2XSSC for 5 min, 50% formamide for 15 min at 40° C, 2XSSC for 15 min, 1XSSC for 15 min and 4XSSC for 5 min for binding of the probe minimal homology. Few gentle shaking was done while washing in 2XSSC and 1XSSC solutions. The DNA slides were covered with parafilm after placing 65µl of antidigoxigenin rhodamine conjugate for digoxigenin labelled 5S rRNA probe and an avidine-FITC (Fluorescein isothiocyanate) conjugate for biotin labelled 18S-5.8S-26S rRNA gene probe incubated in dark at 37° C for 1 hour. The slides were then washed with 4XSSC for 10 min, 4XSSC+0.1% triton X-100 for 10 min, 4XSSC for 10 min and 2XSSC for 5 min All these steps were performed in dark and first three washing were done on orbital shaker 50rpm at room temperature. Subsequently, the slides were rinsed and mounted in a DABCO solution (1.25% DABCO in 90% glycerol) with 2.0ng/µl DAPI as a counterstain. Hybridization signals were observed under the fluorescence microscope. The observation on localization of rDNA sites at metaphase chromosomes were taken into account for final karyotyping.

Results

Karyotypic distribution of rDNA

Fluorescent *in situ* hybridization (FISH) was performed in order to elucidate the number and position of rDNA sites on chromosomes of the standard karyotype of *Bunium persicum*, that was revealed earlier from aceto-carmine stained chromosome plates; DAPI stained chromosomes were taken into account. *In situ* hybridization with biotin labelled

probe pTa71 homologous to 18S-5.8S-26S rDNA detected as green fluorescent signals with fluorescein-conjugated avidin DN. Whereas digoxigenin-labelled 5S rDNA probe of onion detected as red fluorescent signals with rhodamine-conjugated anti-digoxigenin. The visulization of different fluorescent signals facilitated *in situ* chromosomal localization of the respective rDNA sites at varying lengths of chromosomes. It was revealed that there were 2 pairs of 18S-5.8S-26S sites in *Bunium persicum*. One pair of the homologous clusters is located on the telomeric region of the short arm of chromosome 1 and the second on the telomeric region of the short arm of chromosome 2. In chromosome 1, the rDNA site was localized at the secondary constriction region that contained the flanking terminal portion of the short arm, and a satellite, which can be visualised more easily in Figs 1c and 1d. Two pairs of 5S rDNA sites were localized in subtelomeric regions of smaller chromosomes 5 and 7. One 5S rDNA site was localized in the subtelomeric region of the long arm of chromosome 5 and the other 5S rDNA locus was observed at the interstitial region on the short arm of chromosome 7. These loci are shown in Figs 1a and 1b. Fig. 2 depicts the ideogram with the location of rDNA sites.

Discussion

It is evident from the previous studies that rDNA loci are variable in numbers and locations in different crop (Mukai et al. 1990, 1991 in common wheat; Irifune et al. 1995 and Seo et al. 1999 in *Allium* Linnaeus, 1753 species; Abirached Darmenci 2005 in *Medicago truncatula* Gaertn., 1791; Fukui et al. 1994 in *Oryza* Linnaeus, 1753 and Lavania et al. 2005 in *Chlorophytum* Ker Gawler, 1877). Ribosomal RNA (rRNA) multigene families consist of the 18S-5.8S-26S and 5S rRNA genes. In some eukaryotes such as yeast and moss, the 5S and 18S-5.8S-26S rRNA genes are in juxtaposition in the same locus, whereas in other eukaryotes, they are organized as families of

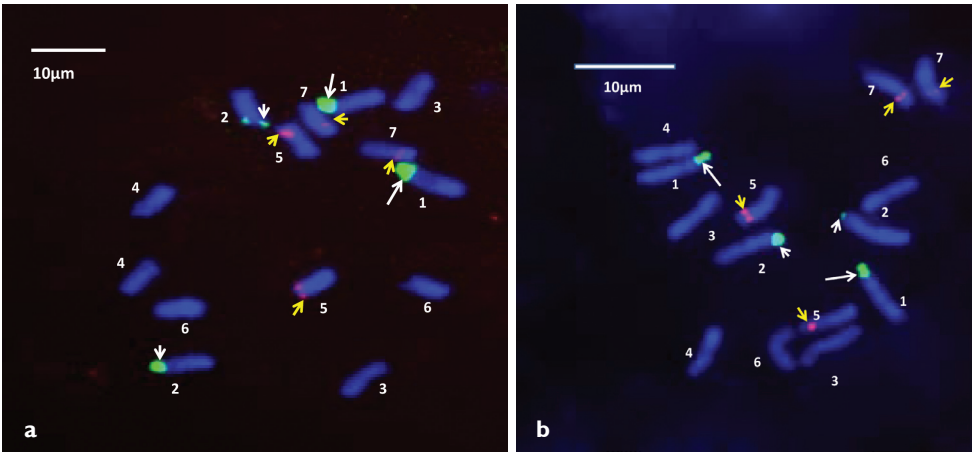

Figure 1a, b. FISH on metaphase chromosomes of *Bunium persicum* using probes of 18S-5.8S-26S indicated by white arrows (NOR (white long arrows), and 5S rRNAgenes (small yellow arrows).

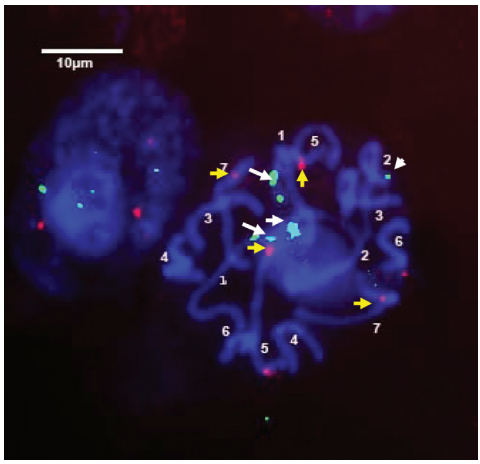

Figure 1c. FISH on Prophase chromosomes of *Bunium* using probes of 18S-5.8S-26S indicated by white arrows (NOR (white long arrows), and 5S rRNAgenes (small yellow arrows).

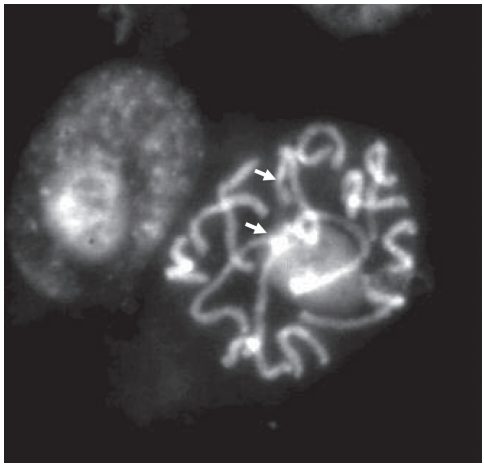

Figure 1d. Prophase chromosome of *Bunium persicum* with DAPI to visualise the NOR regions (white arrows).

tandemly repeated units located at one or a few chromosomal sites (Flavell 1986) and may be unlinked on the same chromosome arm or located on different chromosomes (Mukai et al. 1990). Visualization of the 5S and 18S-5.5S-26S rRNA genes by FISH has provided a number of chromosomal markers to elucidate the chromosomal evolution and species interrelationships (Mukai 2005). In the past decades, FISH studies have been conducted in numerous plant species to elucidate the number and localization of rDNA sites. It has been observed that most of the diploid plants have two sites (i.e. a single locus) of both 5S and 18S-5.8S-26S rDNA (Mishima et al. 2002), although some diploids may have multiple sites (Fukui et al. 1994; Badaeva et al. 1996; De Bustos et al. 1996; Raina and Mukai 1999; Lavania et al. 2005). Prokopowich et al. (2003), based on exhaustive analysis on the rDNA copy number and genome size

in large number of animal and plant taxa, have suggested a strong positive correlation between genome size and rDNA copy number. This helped us to understand that ribosomes would increase as genome size increases if the relative proportion of protein-coding genes remains constant.

In *Bunium persicum* all chromosomes were arranged according to the length, morphology and chromosomal markers (18S-5.8S-26S and 5S rDNA), with the largest chromosome designated as the chromosome 1 and smallest as the chromosome 7 (Fig. 2). The majority of the chromosomes were metacentric making identification of homologous pairs difficult as the size gradient and morphology of chromosomes 5 and 7 and chromosomes 4 and 6 was conspicuous. Identification of long and short arms on which rDNA loci were located was achieved by comparing the same photographs taken before and after *in situ* hybridization (Figs 1c, d). Slight variation in the long arm of chromosomes 4 and 6 helped us to identify homologous pair of each chromosome. The position of two pairs of 5S rDNA sites at different arms was used to identify homologous pairs of chromosome 5 and 7. On the chromosome 5, the 5S rDNA site was located on long arm at subtelomeric region, whereas it was at the interstitial region of the short arm of chromosome 7. Due to small variation in 5S rDNA sites in chromosomes 5 and 7, FISH analysis was carried out at prophase stage to identify the exact location of 5S rDNA sites in these chromosomes (Fig. 1c). The present study revealed that there were two sites for 18S-5.8S-26S rDNA loci each at telomeric regions on chromosomes 1 and 2. Therefore, the identification of chromosomes 1 and 2 was ascertained by visualizing 18S-5.8S-26S rrDNA signals. The 18S-5.8S-26S rDNA signals were over a longer distance on chromosome 1, indicating the presence of secondary constriction (NOR) and a satellite of the chromosome. Some authors have reported that the 18S-5.8S-26S rRNA multigene family, as a compo-

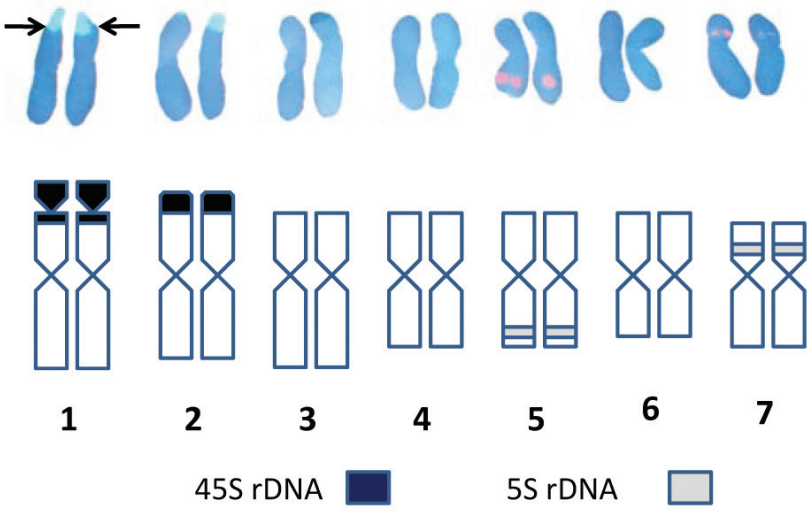

Figure 2. Karyo-idiograms of *Bunium persicum* showing FISH based localization of two rDNA sites (18S-5.8S-26S and 5 S rDNA) on somatic chromosomes of *Bunium persicum.*

nent of the nucleolar organizing region (NOR) which is strongly hybridized to the secondary constriction and satellite (Mukai et al. 1991; Hizume 1994; Castilho and Heslop-Harrison 1995). Although the satellite identification by conventional staining is very difficult because of its small and fragile constriction site, FISH signal allowed the identification of chromosomes with similar morphology. Therefore, the chromosomes 1 and 2 despite having the same size and presence of 18S-5.8S-26S rDNA loci at terminal regions could be discriminated by the long FISH signals on chromosome 1. The bi-or multicolour FISH technique, using rRNA multigene families and other detectable DNA sequences as probes will be useful for determining the marker chromosomes that are similar in size and morphology among species. The present study revealed that chromosomes 5 and 7 were marker chromosomes for 5S rRNA gene and that chromosomes 1 and 2 were marker chromosomes for18S-5.8S-26S rRNA gene. The establishment of the karyotype for *Bunium persicum* may allow the assignment of linkage groups by FISH. This will help to undertake further cytogenetic studies and physical mapping of the loci that can act as landmarks source for the development of genetic map in this plant.

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Karyotypes, male meiosis and comparative FISH mapping of 18S ribosomal DNA and telomeric $(TTAGG)_n$ repeat in eight species of true bugs (Hemiptera, Heteroptera)

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Abstract

Eight species belonging to five true bug families were analyzed using DAPI/CMA_3-staining and fluorescence *in situ* hybridization (FISH) with telomeric $(TTAGG)_n$ and 18S rDNA probes. Standard chromosomal complements are reported for the first time for *Deraeocoris rutilus* (Herrich-Schäffer, 1838) (2n=30+2m+XY) and *D. ruber* (Linnaeus, 1758) (2n=30+2m+XY) from the family Miridae. Using FISH, the location of a 18S rDNA cluster was detected in these species and in five more species: *Megaloceroea recticornis* (Geoffroy, 1785) (2n=30+XY) from the Miridae; *Oxycarenus lavaterae* (Fabricius, 1787) (2n=14+2m+XY) from the Lygaeidae s.l.; *Pyrrhocoris apterus* (Linnaeus, 1758) (2n=22+X) from the Pyrrhocoridae; *Eurydema oleracea* (Linnaeus, 1758) (2n=12+XY) and *Graphosoma lineatum* (Linnaeus, 1758) (2n=12+XY) from the Pentatomidae. The species were found to differ with respect to location of a 18S rRNA gene cluster which resides on autosomes in *O. lavaterae* and *P. apterus*, whereas it locates on sex chromosomes in other five species. The 18S rDNA location provides the first physical landmark of the genomes of the species studied. The insect consensus telomeric pentanucleotide $(TTAGG)_n$ was demonstrated to be absent in all the species studied in this respect, *D. rutilus*, *M. recticornis*, *Cimex lectularius* Linnaeus, 1758 (Cimicidae), *E. oleracea*, and *G. lineatum*, supporting the hypothesis that this motif was lost in early evolution of the Heteroptera and secondarily replaced with another motif (yet unknown) or the alternative telomerase-independent mechanisms of telomere maintenance. Dot-blot hybridization

analysis of the genomic DNA from *C. lectularius*, *Nabis* sp. and *O. lavaterae* with $(TTAGG)_n$ and six other telomeric probes likewise provided a negative result.

Keywords
Karyotypes, meiosis**,** FISH, 18S rDNA, telomeres, dot-blot*,* *Deraeocoris*, *Megaloceroea*, *Nabis, Cimex*, *Oxycarenus*, *Pyrrhocoris*, *Eurydema*, *Graphosoma*, Heteroptera

Introduction

Fluorescence *in situ* hybridization (FISH), established in the 1980s, represents a powerful cytogenetic technique for a visualization of specific DNA sequences onto chromosomes, generating detailed chromosome mapping of eukaryote genomes (Pinkel et al. 1986). Despite the fact that the FISH mapping of insect chromosomes has been under way for a number of years (reviewed by Frydrychová et al. 2004; Vítková et al. 2005), the information of this sort for true bugs is still very scanty and available only for few species studied in respect to telomeric sequences (Okazaki et al. 1993; Sahara et al. 1999) and the location of ribosomal RNA genes (Cattani et al. 2004; Severi-Aguiar et al. 2005, 2006; Papeschi and Bressa 2006; Morielle-Souza and Azeredo-Oliveira 2007; Bressa et al. 2008, 2009; Panzera et al. 2010; Grozeva et al. 2010; Bardella et al. 2010; Poggio et al. 2011).

To fill this gap and learn more about bug genomes, we applied FISH technique with telomeric $(TTAGG)_n$ and 18S rDNA probes to eight species belonging to 7 genera, 5 families and 2 infrforders: *Deraeocoris ruber* (Linnaeus, 1758), *D. rutilus* (Herrich-Schäffer, 1838), and *Megaloceroea recticornis* (Geoffroy, 1785) from the family Miridae, *Cimex lectularius* Linnaeus, 1758 from the family Cimicidae (all from the infraorder Cimicomorpha); *Oxycarenus lavaterae* (Fabricius, 1787) from the family Lygaeidae s.l., *Pyrrhocoris apterus* (Linnaeus, 1758) from the family Pyrrhocoridae, *Eurydema oleracea* (Linnaeus, 1758) and *Graphosoma lineatum* (Linnaeus, 1758) from the family Pentatomidae (all from the infraorder Pentatomomorpha). The 18S rDNA location provided the first physical landmark of the genomes of the species studied. The species *D. ruber* and *D. rutilus* were studied here for the first time likewise in terms of their standard chromosomal complements.

In five species, *M. recticornis*, *D. rutilus*, *C. lectularius*, *E. oleracea*, and *G. lineatum*, we used a $(TTAGG)_n$ telomeric probe to justify a hypothesis that this telomeric motif is absent in the true bugs (Frydrychová et al. 2004). The last hypothesis has been so far based only on studies of two species, *Halyomorpha halys* (Stål, 1855) (Okazaki et al. 1993: as *Halyomorpha mista* (Uhler, 1860)) and *Pyrrhocoris apterus* (Sahara et al. 1999) that do not adequately represent the diversity of the Heteroptera.

Additionally, we carried out a dot-blot hybridization of the genomic DNA from *Cimex lectularius*, *Nabis* sp. and *Oxycarenus lavaterae* with seven types of telomeric probes, ciliate $(TTTTGGGG)_n$ and $(TTGGGG)_n$, nematode $(TTAGGC)_n$, insect $(TTAGG)_n$, shrimp $(TAACC)_n$, vertebrate $(TTAGGG)_n$, and plant $(TTTAGGG)_n$.

Material and methods

Insects

Adult males of *D. ruber*, *Deraeocoris rutilus*, *Megaloceroea recticornis*, *Nabis* sp., *Cimex lectularius*, *Oxycarenus lavaterae*, *Pyrrhocoris apterus*, *Eurydema oleracea*, and *Graphosoma lineatum* were collected in the vicinities of Plovdiv and Sofia, Bulgaria in 2009-2011 (Table 1). On capture, specimens were immediately fixed in a Carnoy fixative (3 parts of 96% ethanol and 1 part of glacial acetic acid) and stored at 4°C until required.

Preparations

The gonads were dissected out and squashed in a drop of 45% acetic acid. The cover slip was removed using the dry ice. Slides were dehydrated in fresh fixative and air dried. The preparations were first analyzed with a phase contrast microscope at 400x. The best chromosome spreads were used for different staining techniques.

Table 1. Material analyzed

Infraorder, family, and species	Locality in Bulgaria	Date of collection	Number of specimens analyzed
Cimicomorpha			
Miridae			
Deraeocoris ruber	Bulgaria, Western Rhodopes Mts., near Kuklen Vill., 42.032990°N, 024.774537°E, 384 m a.s.l.	9.06.2009	2
Deraeocoris rutilus	Bulgaria, Western Rhodopes Mts., near Kuklen Vill., 42.032990°N, 024.774537°E, 384 m a.s.l.	8.06.2009	2
Megaloceroea recticornis	Bulgaria, Asenovgrad, 42.05977°N, 024.813424°E, 177m a.s.l.	9.06.2009	12
Nabidae			
Nabis sp.	Bulgaria, Sofia, City Center	15.06.2010	4
Cimicidae			
Cimex lectularius	Bulgaria, Sofia, Studentski Grad	14.10.2010	3
Pentatomomorpha			
Lygaeidae			
Oxycarenus lavaterae	Bulgaria, Sofia, City Center, on *Tilia* sp.	3.07.2011	4
Phyrrocoridae			
Pyrrhocoris apterus	Bulgaria, Sofia, City Center, on *Tilia* sp.	3.07.2011	2
Pentatomidae			
Eurydema oleracea	Bulgaria, Western Rhodopes Mts., near Progled Vill., 41.68067°N, 024,70527°E, 1320 m a.s.l.	9.06.2009	2
Graphosoma lineatum	Bulgaria, Thracian Lowland, outflow of Chaya River in Maritsa River, 42.147653°N, 024,880186°E, 152 m a.s.l.	8.06.2009	3

Fluorochrome banding

To reveal the base composition of C-heterochromatin, staining by GC-specific chromomycin A_3 (CMA_3) and AT- specific 4-6-diamidino-2-phenylindole (DAPI) was used according to Schweizer (1976) and Donlon and Magenis (1983) respectively, with some modifications. C-banding pretreatment was first carried out using 0.2 N HCl at room temperature for 30 min, followed by 7-8 min treatment in saturated $Ba(OH)_2$ at room temperature and then an incubation in 2xSSC at 60°C for 1 h. Furthermore, the preparation (without Giemsa) were stained first with CMA_3 (0.4 μg/ml) for 25 min and then with DAPI (0.4 μg/ml) for 5 min. After staining, the preparations were rinsed in the McIlvaine buffer, pH 7 and mounted in an antifade medium (700 μl of glycerol, 300 μl of 10 mM McIlvaine buffer, pH 7, and 10 mg of N-propyl gallate).

Fluorescence *in situ* hybridization (FISH)

DNA isolation, PCR amplification, probe generation

Genomic DNA from a male of *Pyrrhocoris apterus* (Heteroptera, Pyrrhocoridae) was isolated using a Chelex-100 extracted method. FISH using a 18S rRNA gene probe was carried out on the chromosomes of *D. ruber*, *D. rutilus*, *M. recticornis*, *O. lavaterae*, *P. apterus*, *E. oleracea*, and *G. lineatum.* FISH using a telomeric $(TTAGG)_n$ probe was carried out on the chromosomes of *D. rutilus*, *M. recticornis*, *C. lectularius*, *E. oleracea*, and *G. lineatum*. The target 18S rDNA gene was PCR amplified (primers presented in Table 2) from the genomic DNA of *P. apterus*, and labeled by PCR with biotin. Telomere probe $(TTAGG)_n$ was PCR amplified and labeled using primers TTAGG_F and TTAGG_R (Table 2) and Rhodamine-5-dUTP (GeneCraft, Germany).

FISH procedure

In situ hybridization was performed as described by Schwarzacher and Heslop-Harrison (2000) with modifications. In each species, one or two FISH preparations were examined. Chromosome preparations were dehydrated through 70/80/96% Ethanol at RT and treated with 100 μg/ml RNaseA (Sigma) for 60 min at 37°C in a humid chamber; washed three times in 2x SSC (5 min each) at RT; dehydrated through 70/80/96% Ethanol at RT; incubated in 5 mg/ml Pepsin in 0.01 N HCl for 15 min at 37°C; washed sequentially in 1x PBS, in PBSx1/0.05M $MgCl_2$ for 5 min each, in 1% PFA in PBSx1/0.05M $MgCl_2$ for 10 min, in 1x PBS for 5 min, in PBSx1/0.05M $MgCl_2$ for 5 min at RT each; dehydrated through 70/80/96% Ethanol at RT or ice cold and finally, dried. After pretreatment, hybridization mixture containing about 100 ng of labeled probe, 50% formamide, 2×SSC, 10% (w/v) dextran sulfate, 1% (w/v) Tween-20 and 10 μg salmon-sperm DNA was added on preparations. Slides were mounted using glass coverslips and rubber

Table 2. PCR primers used in present study

Name	Sequence (5‘ – 3‘)
18S_F	ACAAGGGGCACGGACGTAATCAAC
18S_R	CGATACGCGAAT GGCTCAAT
Eup_F	TTTTGGGGTTTTGGGGTTTTG
Eup_R	CCCCAAAACCCCAAAACCC
Prot_F	TTGGGGTTGGGGTTGGGG
Prot_R	CCCCAACCCCAACCCCAA
Wrm_F	TTAGGCTTAGGCTTAGGCTT
Wrm_R	GCCTAAGCCTAAGCCTAAG
TTAGG_F	TAACCTAACCTAACCTAACCTAA
TTAGG_R	GGTTAGGTTAGGTTAGGTTAGG
Shr_F	TAACCTAACCTAACCTAACCTAA
Shr_R	GGTTAGGTTAGGTTAGGTTAGG
TTAGGG_F	CCCTAACCCTAACCCTAACCCTAACCCTAA
TTAGGG_R	TTAGGGTTAGGGTTAGGGTTAGGGTTAGGG
Plnt_F	TTTAGGGTTTAGGGTTTAGGG
Plnt_R	CCCTAAACCCTAAACCCTAAA

cement. The slides were denaturated for 5 min at 75°C. Then the chromosome slides were incubated for 42–44 h at 37°C. Following hybridization, the slides were washed in 2x SSC for 3 min at 45°C, then in 50% formamide in 2xSSC for 10 min at 45°C, two times in 2x SSC (10 min each) at 45°C, blocked in 1.5% (w/v) BSA/4x SSC/0.1% Tween-20 for 30 min at 37° in a humid chamber. 18S rRNA gene probe was detected with 5μg/ml Avidin-Alexa Fluor 488 (Invitrogen). Detection reaction was performed in 1.5 % BSA/ 4x SSC/0.1% Tween-20 for 1 h at 37°C. Slides were washed three times in 4x SSC/0.02% Tween-20 (10 min each) at 45° and dehydrated through 70/80/96% ethanol at RT. Chromosomes were mounted in a mounting-antifade (ProLong Gold antifade reagent with DAPI, Invitrogen) and covered with a glass coverslip.

Dot-blot analysis

Genomic DNA from *Cimex lectularius*, *Oxycarenus lavaterae* and *Nabis* sp. was isolated using NucleoSpin Tissue Kit (Macherey-Nagel, Germany) or a standard Phenol/Chloroform nucleic acid extraction protocol. Telomere probes of ciliate $(TTTTGGGG)_n$ and $(TTGGGG)_n$, nematode $(TTAGGC)_n$, insect $(TTAGG)_n$, shrimp $(TAACC)_n$, vertebrate $(TTAGGG)_n$ and plant $(TTTAGGG)_n$ were PCR amplified using primers labeled with biotin and presented in Table 2.

About 20 ηg of isolated DNA after denaturation was added drop wise to Hybond N+ nylon membranes (Amersham, Biosciences). Hybridizations were carried out over night in hybridization mixture containing about 100-200 ηg of labeled probe, 50% formamide, 4×SSC, 0.5% (w/v) SDS and 10 μg salmon-sperm DNA at 40 °C. Mem-

branes were washed two times in 2x SSC/0.1% SDS (10 min each) at RT and two times in 0.2x SSC/0.1% SDS (10 min each) at RT (10 min each). Detection procedure was performed according to the Biotin Chromogenic Detection Kit protocol (Fermentas).

Microscopy and imaging

Chromosome preparations were analyzed under a Leica DM 4000B microscope with a 100x objective. Fluorescence images were taken with a Leica DFC 350 FX camera using Leica Application Suite 2.8.1 software with an Image Overlay module. The preparations were stored partly at Institute of Biodiversity and Ecosystem Research, BAS in Sofia and partly at the Zoological Institute, RAS in St Petersburg.

Results

Miridae

***Deraeocoris ruber* Linnaeus, 1758,** 2n=30+2m+XY
Fig. 1a–d

The karyotype is described here for the first time. There are 16 bivalents, including a pair of very small and negatively heteropycnotic chromosomes taken as a pair of m-chromosomes, and the univalent X and Y chromosomes which are largest and smallest chromosomes in the set, respectively (Fig. 1a–d). Diplotene and diakinesis stages were not detected, and bivalents displayed no chiasmata since meiosis is achiasmate. 18S rRNA genes were mapped on both sex chromosomes, the signals being more intensive on the Y. At spermatogonial metaphases, a number of very small intercalary signals could be in addition seen on the X (Fig. 1a).

***Deraeocoris rutilus* (Herrich-Schäffer, 1838),** 2n=30+2m+XY
Fig. 2a, b

The karyotype is described here for the first time. It is much like that described above for *D. ruber*. Likewise, at PMI, there are 16 autosomal bivalents and the univalent X and Y chromosomes. One of the bivalents is very small, negatively heteropycnotic pair of m-chromosomes (Fig. 2a, b). The X is the largest and the Y is one of the smallest chromosomes in the set (excluding the m-chromosomes); autosomal bilvalents constitute a decreasing size raw. Diplotene and diakinesis stages were not observed, and meiosis was considered achiasmate of a collochore type.

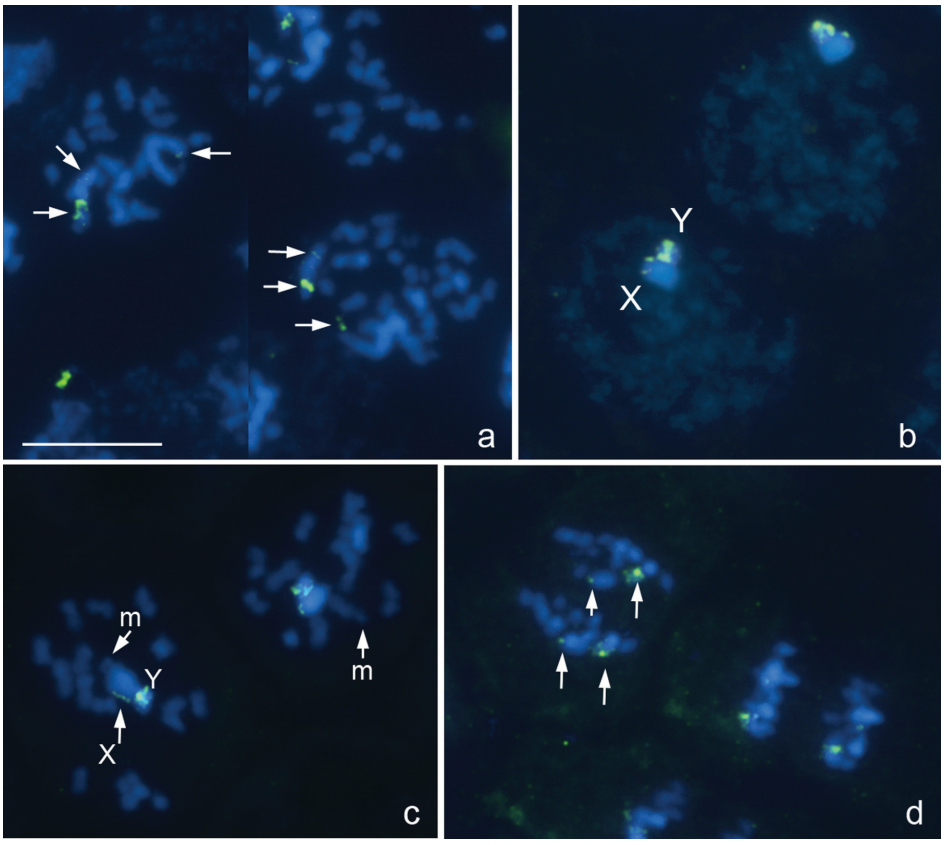

Figure 1a–d. *Deraeocoris ruber*, 2n=30+2m+XY. **a** spermatogonial prometaphase **b** early prophase **c** prometaphase I **d** anaphase II. FISH with an 18S rDNA probe. Arrowed are 18S rDNA clusters **a, d** Bar equals 10 μm.

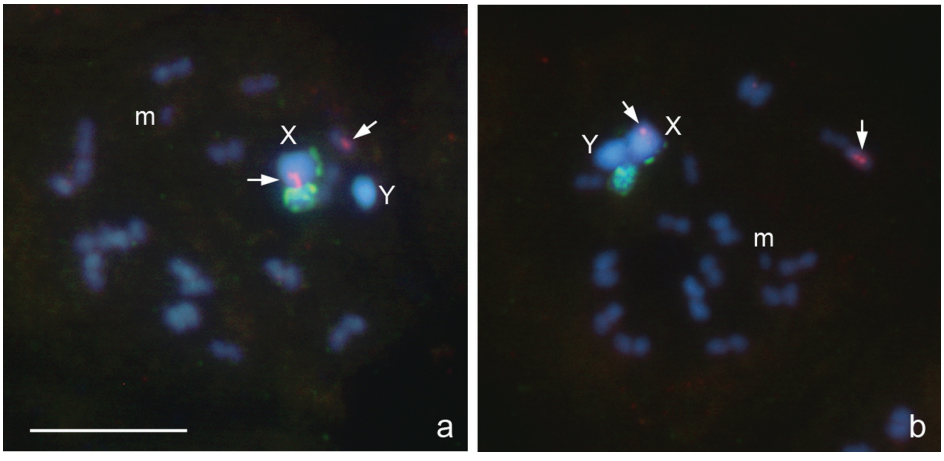

Figure 2a, b. *Deraeocoris rutilus*, 2n=30+2m+XY. **a, b** prometaphase I. FISH with 18S rDNA and $(TTAGG)_n$ probes. Arrowed are signals after using a TTAGG-probe. Bar equals 10 μm.

FISH with an 18S rDNA probe produced two local signals placed near a telomeric region of the X chromosome and in addition a huge cluster of signals attached to the X; Y chromosome carried no signal (Fig. 2a, b). FISH with a TTAGG probe produced prominent hybridization signals (Fig. 2a, b, arrowed), which were occasionally located on the same chromosomes however most likely did not indicate the telomeres.

***Megaloceroea recticornis* (Geoffroy, 1785),** 2n=30+XY
Fig. 3a–f

From the counts of 23 plates at prometaphase I (PMI), 15 autosomal bivalents and two univalent sex chromosomes, X and Y, were detected suggesting that this species displays 2n=30+XY (Fig. 3a -c) in contrast to 2n=32+XY previously reported for this species in England (Leston 1957). The X is fairly large whereas the Y is one of the smallest chromosomes in the set, and the autosomal bilvalents constitute a decreasing size raw. There are no visible constrictions in the chromosomes, since they are holokinetic. During meiotic prophase, the diplotene and diakinesis stages escaped detection. At condensation stage, the bivalents showed no chiasmata however homologues were connected with each other by tenacious thread-like structures, at least, at one site, and the telomeric regions pushed off from each other (Fig. 3a, b). Taken together, the observations of meiosis suggest this species to display the achiasmate meiosis of a collochore type. Fluorescence *in situ* hybridization with a $(TTAGG)_n$ probe did not reveal positive signals on the telomeres although occasionally gave rise to variable interstitial hybridization signals, sometimes quite bright, on separate chromosomes (Fig. 3f). Major ribosomal DNA cistrons were shown to locate on both X and Y chromosomes as detected by FISH with a 18S rDNA probe (Fig. 3d–f). At MII, X and Y chromatids associate forming an XY pseudo-bivalent (Fig. 3f) and segregate reductionally. MI plates are nonradial with X and Y chromosomes distributed among the bivalents (Fig. 3c), whereas MII plates are clearly radial, and XY pseudo-bivalent is located at the center of the ring formed by autosomes (Fig. 3f).

Cimicidae

***Cimex lectularius* Linnaeus, 1758,** $2n=26+X_1X_2Y$
not figured

This study confirms that *Cimex lectularius* display $2n=26+X_1X_2Y$ as it was repeatedly reported previously (see Grozeva et al. 2010 and references therein). FISH with a TTAGG probe produced no signals on the chromosome spreads (not shown).

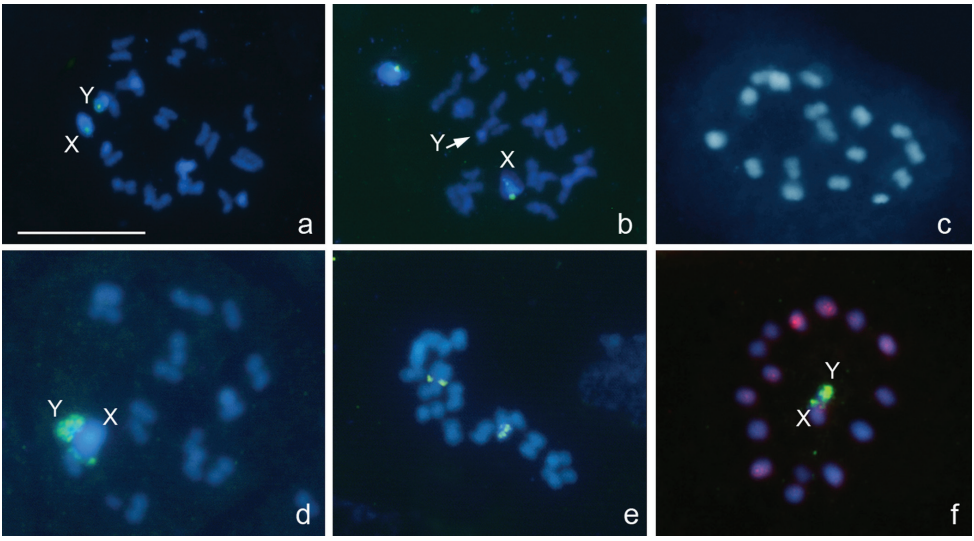

Figure 3a–f. *Megaloceroea recticornis*, 2n=30+XY. **a, b, d** condensation stage; **c, e** prometaphase I; **f** metaphase II. FISH with 18S rDNA **a, b, d–f** and $(TTAGG)_n$ **f** probes. Bar equals 10 μm.

Lygaeidae s.l.

***Oxycarenus lavaterae* (Fabricius, 1787)**, 2n=14+2m+XY
Fig. 4a, b

In accordance with earlier published data (Grozeva 2004), the karyotype of this species includes 18 chromosomes as evidenced by a spermatogonial metaphase (Fig. 4a) and meiotic MI with 8 autosomal bivalents and univalent X and Y chromosomes (Fig. 4b). One of the bivalents is very small and negatively heteropycnotic and taken as a pair of m-chromosomes described earlier in six other species of the subfamily Oxycareninae (Grozeva 1995). The m-chromosomes are likewise well recognized at the spermatogonial metaphase (Fig. 4a). The 18S rDNA signals could be easily seen on the second largest pair of autosomes (Fig. 4a, b).

Pyrrocoridae

***Pyrrhocoris apterus* (Linnaeus, 1758),** 2n=22+X
Fig. 5a–f

In accordance with previously published data (Henking 1891, Ueshima 1979, Sahara et al. 1999), the species displays 2n=22+X in males as indicated by our observations of different stages of meiosis (Fig. 5c–f). FISH with an 18S rDNA probe produced clear interstitial signals on every homologue of a larger autosomal bivalent best demonstrated in Figures 5c and 5d. Figure 5b (meiotic prophase) shows that signals are present on

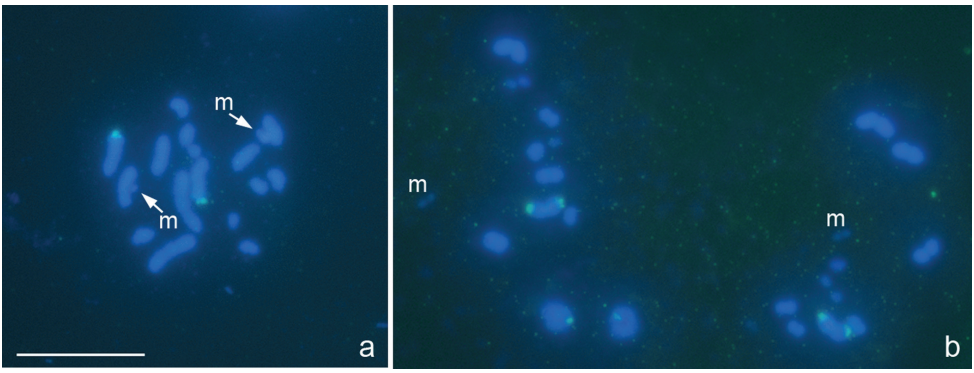

Figure 4a, b. *Oxycarenus lavaterae*, 2n=14+2m+XY. **a** spermatogonial metaphase **b** metaphase I. FISH with a 18S rDNA probe. Bar equals 10 μm.

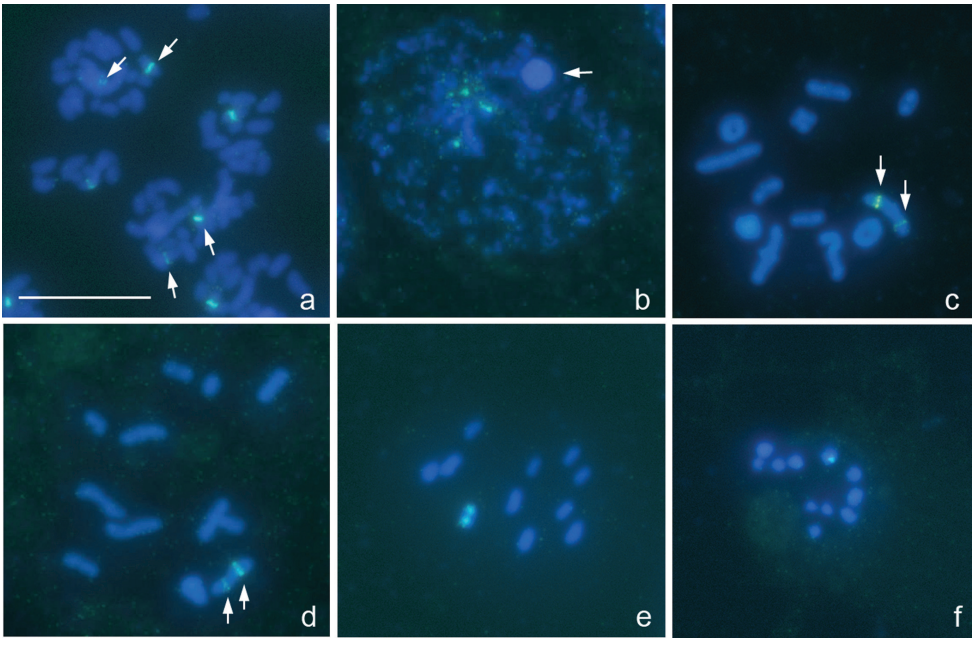

Figure 5a–f. *Pyrrhocoris apterus*, 2n=22+X. **a** spermatogonial prometaphase **b** early prophase (arrowed is the sex chromosome body) **c, d** prometaphase I **e** metaphase II **f** telophase II. FISH with an 18S rDNA probe. Arrowed are 18S rDNA clusters. Bar equals 10 μm.

autosomes and absent on a sex chromosome body (arrowed). We call attention to difference in signal strength between the homologues (Fig. 5a, c, d), which is most likely caused by difference in 18S rRNA gene copy number.

Pentatomidae

***Eurydema oleracea* (Linnaeus, 1758)**, 2n=12+XY
Fig. 6a–f

This study confirms that *E. oleracea* has 2n=12+XY as previously reported by other researchers (Schachow 1932, Geitler 1939, Xavier and Da 1945). At different prophase stages and at MI (Fig. 6a–d), 6 autosomal bivalents and two univalent sex chromosomes, X and Y, were observed. The chromosomes are fairly large as compared with those in the above mentioned multichromosomal species. In *E. oleracea*, the X chromosome is medium-sized whereas the Y chromosome is the smallest in the set; autosomal bilvalents constitute a decreasing size raw. FISH with a $(TTAGG)_n$ probe did not reveal positive signals on chromosomal spreads. Clear 18S rDNA signals were evident on both sex chromosomes (Fig. 6a–d). Results of fluorochrome staining were consistence with the FISH evidence since CMA_3-positive/DAPI-negative regions were observed on the sex chromosomes confirming thus the presence here the ribosomal loci (Fig. 6e, f). In meiosis, both MI and MII plates were radial with univalent sex chromosomes at MI (Fig. 6d) and an X and Y pseudo-bivalent at MII (not shown) being located at the centre of the ring formed by autosomes. A number of MI plates demonstrated the deviations from the radiality with some of the autosomal bivalents lying at the center of the ring or one of sex chromosomes lying outside the ring.

***Graphosoma lineatum* (Linnaeus, 1758)**, 2n=12+XY
Fig. 7a–d

The karyotype of 2n=12+XY discovered here in *G. lineatum* is in accordance with that published previously for other populations of this species (Geitler 1939, Xavier and Da 1945). The karyotype closely parallels that in *E. oleracea*. The chromosomes are fairly large and noticeably larger as compared with the multichromosomal species. At MI, there are 6 bivalents and the univalent X and Y chromosomes, the X chromosome being medium-sized and the Y the smallest chromosome in the set; autosomal bivalents constitute a decreasing size raw (Fig. 7a–d). FISH with a $(TTAGG)_n$ probe did not reveal positive signals on the chromosomal spreads. Ribosomal DNA cistrons were found to locate at the terminal position on the X as detected by FISH with an 18S rDNA probe (Fig. 7a, b) and DNA binding fluorochromes which revealed DAPI-dull/CMA_3-bright bands on the X (Fig. 7c, d).

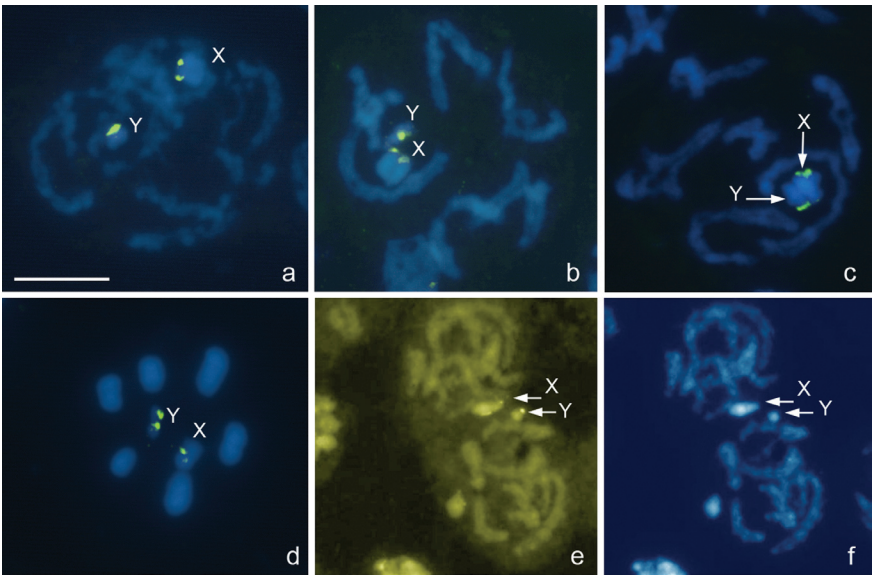

Figure 6a–f. *Eurydema oleracea*, 2n=12+XY. **a-c, e, f** different prophase stages **d** metaphase I. FISH with a 18S rDNA probe **a-d** and CMA_3 /DAPI **e/f**-staining. Bar equals 10 μm.

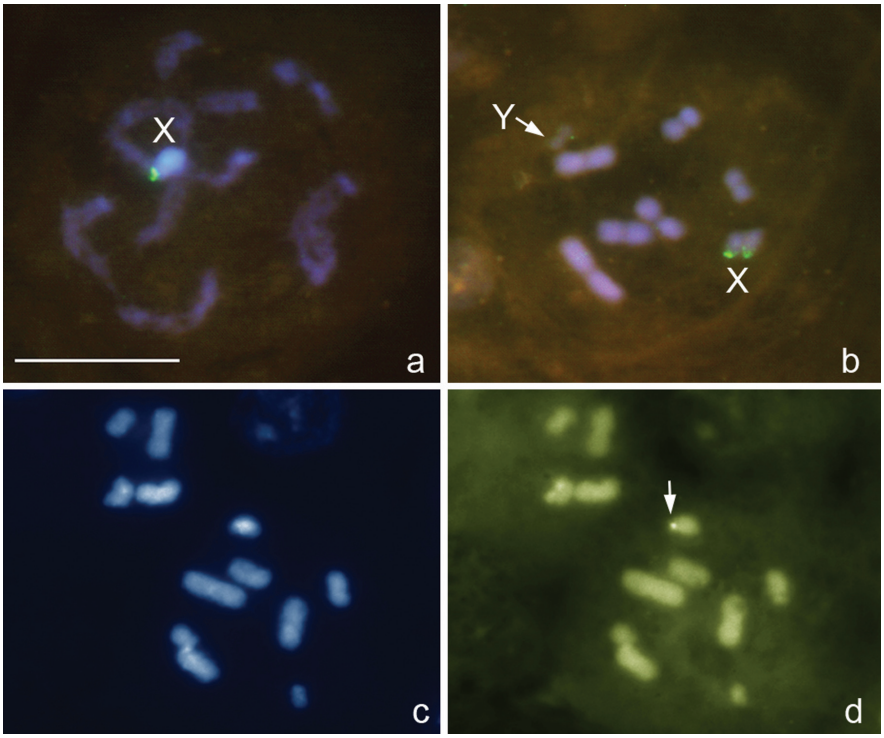

Figure 7a–d. *Graphosoma lineatum*, 2n=12+XY. **a** prophase stage **b–d** metaphase I. FISH with 18S rDNA and $(TTAGG)_n$ probes **a, b** and DAPI **c** and CMA_3 **d**-staining. Arrowed **d** is a CMA_3-positive signal on the X. Bar equals 10 μm.

Dot-blot hybridization analysis

Dot-blot hybridization of the genomic DNA from *Cimex lectularius*, *Nabis* sp. and *Oxycarenus lavaterae* was performed using seven telomeric probes, ciliate $(TTTTGGGG)_n$ and $(TTGGGG)_n$, nematode $(TTAGGC)_n$, insect $(TTAGG)_n$, shrimp $(TAACC)_n$, vertebrate $(TTAGGG)_n$ and plant $(TTTAGGG)_n$. All the experiments provided no hybridizing bands clearly suggesting some other molecular composition of telomeres in true bugs.

Discussion

Standard chromosomal complements

We studied standard chromosomal complements of eight species from 6 genera and 5 families of the true bug infraorders Cimicomorpha (*Deraeocoris rubber*, *Deraeocoris rutilus*, *Megaloceroea recticornis*, *Cimex lectularius*) and Pentatomomorpha (*Oxycarenus lavaterae*, *Pyrrhocoris apterus*, *Eurydema oleracea*, *Graphosoma lineatum*). Our study confirms the previously published information (see Results and Table 3 for the references) that *C. lectularius* (Cimicidae) displays 2n=26+X_1X_2Y; *P. apterus* (Pyrrhocoridae) – 2n=22+X; *O. lavaterae* (Ligaeidae) – 2n=14+2m+XY; *E. oleracea* and *G. lineatum* (Pentatomidae) – 2n=12+XY. On the other hand, Leston (1957) recorded *M. recticornis* (Miridae) in England as having 2n=32+XY; however this count was not corroborated by our observations of this species. The karyotype of *M. recticornis* in Bulgaria, as revealed in our work, is 2n=30+XY. We can not explain this incompatibility, especially as Leston provided neither photograph nor drawing of the chromosomal complement. It should be mentioned here that 2n=32+XY is the first whereas 2n=30+XY the second commonest karyotype in the Miridae (Kuznetsova et al. 2011). The chromosomal complements of *D. rutilus* and *D. ruber* were studied herein for the first time. These species were found to agree with one another in a karyotype of 2n=32+XY, with a pair of m-chromosomes among autosomes, and the karyotype formula is hence determined as 2n=30+2m+XY. A pair of chromosomes (the autosomes) known as m-chromosomes has been described in karyotypes of many bug species (Ueshima 1979). These chromosomes are typically extremely small, negatively heteropycnotic and behave differently as compared to autosomes and sex chromosomes during meiosis. However their origin and significance in genomes remain still obscure. The presence or absence of m-chromosomes seems to represent a fairly stable character at higher taxonomic levels in the Heteroptera (Ueshima 1979). Until the present time, m-chromosomes have been discovered in as few as two Miridae species, *Capsus ater* (Linnaeus, 1758) and *Dicyphus digitalidis* Josifov, 1958 (Nokkala and Nokkala 1986, Grozeva and Simov 2008 a), even though dozens Miridae species were studied in respect to karyotypes (see review: Kuznetsova et al. 2011). Thus, *D. rutilus* and *D. ruber* from our study increased the total number of mirid species with m-chromosomes to four. It is worthy of note that m-chromosomes were not described in the ten previously studied representatives of the genus *Deraeocoris* Kirschbaum,

Table 3. Chromosomal complements and 18S DNA locations in the species studied

Infraorder, family, and species	2n (♂)	Karyotype formula	18S rDNA location	Published data on karyotype
Cimicomorpha				
Miridae				
Deraeocoris ruber	34	2n=30+2m+XY	X and Y chromosomes	Absent
Deraeocoris rutilus	34	2n=30+2m+XY	X chromosome	Absent
Megaloceroea recticornis	32	2n=30+XY	X and Y chromosomes	2n=32+XY (Leston 1957)
Cimicidae				
Cimex lectularius	29	2n=26+X_1X_2Y	X_1 and Y chromosomes*	2n=26+X_1X_2Y (Grozeva et al. 2010 and references therein)
Pentatomomorpha				
Ligaeidae				
Oxycarenus lavaterae	18	2n=14+2m+XY	A pair of larger autosomes	2n=14+2m+XY (Grozeva 2004)
Phyrrocoridae				
Pyrrhocoris apterus	23	2n=22+X	A pair of larger autosomes	2n=22+XX/X0 (Henking 1891, Ueshima 1979, and references therein; Sahara et al. 1999)
Pentatomidae				
Eurydema oleracea	14	2n = 12+XY	X and Y chromosomes	2n=12+XY (Schachow 1932, Geitler 1939, Xavier and Da 1945)
Graphosoma lineatum	14	2n = 12+XY	X chromosome	2n=12+XY (Geitler 1939, Xavier and Da 1945).

*Data from Grozeva et al. 2010

1856 which species were shown to have 2n=32+XY as well (see Ueshima 1979). In some cases m-chromosomes might have been overlooked due to their too small size and negative heteropycnosis in meiosis (Kuznetsova et al. 2011). It remains to be added here that the species studied in this work display holokinetic chromosomes which lack primary constrictions (the centromeres) as in all other Heteroptera (Ueshima 1979).

Male meiosis

In all the seven species studied in this work, the first meiotic division is reductional for the autosomes and equational for the sex chromosomes, and vice versa – the second division is equational for the autosomes and reductional for the sex chromosomes. Such

a behavior of sex chromosomes in male meiosis, or "post-reduction", as it is called, represents one of the unique cytogenetic characters of the Heteroptera being inherent in most bug species (Ueshima 1979). The species studied herein, all except Miridae species, showed the orthodox chiasmate meiosis in males with only one chiasma per bivalent, the meiotic pattern characteristic of holokinetic chromosomes (Halkka 1964, Nokkala et al. 2004). In common with several Miridae species studied so far in this respect ((Nokkala and Nokkala 1986; Grozeva et al. 2006, 2007; Grozeva and Simov 2008a, b), the three mirid species from our work, *Deraeocoris ruber*, *D. rutilus* and *Megaloceroea recticornis*, were found to have achiasmate meiosis of a collochore type (best exemplified by *M. recticornis*). In meiosis of this type, diplotene and diakinesis stages are absent, and no chiasmata are formed between homologous which are however connected with each other, generally only at one site, by thread-like structures, the so-called collochores. The collochores have the function to hold homologous chromosomes together in the absence of chiasmata, and hence ensure their proper orientation and regular segregation at anaphase I.

One of the distinctive properties of the true bug meiosis is a specific spatial arrangement of metaphase plates known as radial ones. Either at both metaphases, MI and MII, or at only one of those, the autosomes (either as bivalents at MI or as univalents at MII) form a ring with the sex chromosomes (either as univalents at MI or as a pseudo-bivalent at MII) lying in its center (Ueshima 1979). In two species studied here on this point, different patterns were observed. The mirid species *Megaloceroea recticornis* displayed MI plates nonradial with X and Y chromosomes distributed among the bivalents, and MII plates clearly radial with XY pseudo-bivalent located at the center of the ring formed by autosomes. Based on our observations of *Eurydema oleracea*, in this pentatomid species both MI and MII plates are radial. We emphasize however that MII plates in this species were more stable in this pattern compared to MI plates, which sometimes demonstrated the deviations from the radiality with some of the autosomal bivalents also lying at the center of the ring or one of sex chromosomes lying outside the ring. The differences between MI and MII in regard to their radial arrangement observed in *E. oleracea* are in agreement with the available data on species from other bug families, including the Pentatomidae (Rebagliati et al. 2003). In another pentatomid species, *G. lineatum*, the first metaphase was nonradial, however there was no MII plates to be analyzed.

Chromosomal location of 18S rDNA clusters

The nucleolus represents a subnuclear compartment of eukaryotic cells in which the synthesis of ribosomal RNA (rRNA) and formation of ribosomes take place (Busch and Smetana 1970). Nucleolar organizer regions (NORs) are usually detected in insects by silver nitrate ($AgNO_3$) and GC-specific fluorochrome (most commonly by CMA_3) staining. However silver treatment stains only active NORs (Hubbell 1985) being therefore inadequate to the study of NOR location onto chromosomes. In con-

trast, fluorescence *in situ* hybridization (FISH) with rDNA probes directly detects the location of ribosomal RNA genes, regardless of their activity. In eukaryotes, *5S* and *18S* ribosomal genes (rDNA) are organized into two multigenic families, namely the major rDNA family formed by the *18S*, *5.8S*, and *28S* genes and the minor one composed of *5S* genes (Long and David 1980). Chromosomal mapping of genes is important for identification of chromosomes, which is especially difficult in groups of organisms with holokinetic chromosomes.

In Heteroptera, physical location of genes remains very poorly sampled (and the data available concern only ribosomal genes), mainly with sporadic sampling of a few select species. Out of more than 40,000 described species (Weirauch and Schuh 2011), approximately 1600 species have been subjected to cytogenetic analysis (Papeschi and Bressa 2006). Among those, only 22 species (11 genera) belonging to the families Reduviidae (Severi-Aguiar et al. 2005, 2006, Morielle-Souza and Azeredo-Oliveira 2007, Bardella et al. 2010, Panzera et al. 2010, Poggio et al. 2011), Cimicidae (Grozeva et al. 2010), Coreidae (Papeschi et al. 2003, Cattani et al. 2004, Bressa et al. 2008), Belostomatidae (Papeschi and Bressa 2006), Pentatomidae (Papeschi et al. 2003), and Pyrrhocoridae (Bressa et al. 2009) have been investigated in respect to FISH rDNA location.

In this study we have characterized the chromosomal locations of 18S RNA genes in seven species from six genera of the families Miridae (*Deraeocoris ruber*, *D. rutilus*, *Megaloceroea recticornis*), Lygaeidae (*Oxycarenus lavaterae*), Pyrrhocoridae (*Pyrrhocoris apterus*), and Pentatomidae (*Eurydema oleracea*, *Graphosoma lineatum*). Data on all the species (as well as those on the whole families Miridae and Lygaeidae) were obtained for the first time bringing thus the total number of the species and genera studied to 30 and 18, respectively. The species were shown to exhibit different patterns of rDNA location. Some of the species showed their ribosomic cistrons located on sex chromosome, either on the X (*D. rutilus*) or on both X and Y (*D. ruber*, *M. recticornis*, *E. oleracea*, *G. lineatum*) whereas in other species (*O. lavaterae*, *P. apterus*) they were located on a pair of autosomes.

The location of 18S rDNA appeared different within the taxa, in which rDNA sequences were mapped in more than one species as in the mirid genus *Deraeocoris*, where *D. rutilus* displayed rDNA clusters concentrated on the X, but *D. ruber* on both X and Y chromosomes. These findings give no way of any inferences especially as a wide variation of chromosomal location for the major rDNA has been observed in different bug taxa, including variations between the co-generic species. For example, the genus *Triatoma* Laporte, 1832 from the subfamily Triatominae (Reduviidae), which is one of the most studied bug groups, clearly shows the interspecific variation (Severi-Aguiar et al. 2005, 2006, Morielle-Souza and Azeredo-Oliveira 2007, Bardella et al. 2010, Poggio et al. 2011) while sometimes even intraspecific variation (Panzera et al. 2010) for the major rDNA harboring either on the sex chromosomes (X and/or Y), or the autosomes or on both. This variability is suggested to be due to the chromosomal exchanges between the autosomes and sex chromosomes during the speciation of the Triatominae (Panzera et al. 2010).

The telomere repeat sequence

The pentanucleotide sequence $(TTAGG)_n$ is known as the commonest and most likely an ancestral DNA motif of insect telomeres (Sahara et al. 1999, Frydrychová et al. 2004). However this motif was lost during the evolution of several groups being secondarily replaced with another motif (yet unknown) or the alternative telomerase-independent mechanisms of telomere maintenance (Frydrychová et al. 2004, Lukhtanov and Kuznetsova 2010). The true bugs are considered as one of the insect higher taxa in which $(TTAGG)_n$ is absent, however the data available concerned so far the two species only, *Halyomorpha halys* (Stål, 1855) (Pentatomidae) studied using Southern hybridization (Okazaki et al. 1993: as *Halyomorpha mista* (Uhler, 1860)) and *Pyrrhocoris apterus* (Pyrrhocoridae) subjected both to Southern hybridization and FISH (Sahara et al. 1999). Comparative analysis of the occurrence of $(TTAGG)_n$ in various groups of insects has showed that this motif is evolutionarily stable, and, having once appeared during evolution, marks taxa and phylogenetic branches of high rank. It is known however that in some groups, such as the orders Coleoptera and Neuroptera, both TTAGG-positive and TTAGG-negative species are encountered (Frydrychová et al. 2004 and references therein). By using FISH we studied the occurrence of $(TTAGG)_n$ telomere repeat in five species: *Deraeocoris ruber* and *Megaloceroea recticornis* (Miridae), *Cimex lectularius* (Cimicidae), *Eurydema oleracea* and *Graphosoma lineatum* (Pentatomidae). All these species were shown to lack the insect consensus sequence. Although in both mirid species a number of prominent hybridization signals could be seen on separate chromosomes, these signals most likely did not indicate the telomeres. The presence of signals suggests a sequence related to TTAGG but it seems to have no target specificity in the bug chromosomes.

The absence of $(TTAGG)_n$ telomeric repeat in the phylogenetically distant groups within the Heteroptera strengthens thus the view (Frydrychová et al. 2004) that it was lost in early evolution of this group of insects.

Dot-blot hybridization of the genomic DNA from bug species with seven types of telomeric probes, ciliate $(TTTTGGGG)_n$ and $(TTGGGG)_n$, nematode $(TTAGGC)_n$, insect $(TTAGG)_n$, shrimp $(TAACC)_n$, vertebrate (TTAGGG)n and plant $(TTTAGGG)_n$, yielded negative results and did not provide hence any answer of the question which is the telomere repeat sequence in bug chromosomes.

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