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First record of supernumerary (B) chromosomes in electric fish (Gymnotiformes) and the karyotype structure of three species of the same order from the upper Paraná River basin

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Abstract

Cytogenetic studies were performed on the species *Apteronotus* prope *albifrons* Linnaeus, 1766, *Rhamphichthys hahni* Meinken, 1937 and *Brachyhypopomus gauderio* Giora & Malabarba, 2009, collected in the upper Paraná River floodplain, Porto Rico (PR), Brazil. *Apteronotus* prope *albifrons* showed a diploid number of 2n=24 chromosomes for both sexes and a karyotype formula of 14m+2sm+2st+6a (FN=42). Besides the standard karyotype, three specimens had one to three extra microchromosomes with inter- and intra-individual variations, which suggested the occurrence of B chromosomes in the species. The chromosomal data of *R. hahni*, described here for the first time, consists of 50 chromosomes and a formula comprised of 20m+24sm+6a (FN=94). *Brachyhypopomus gauderio* specimens demonstrated 2n=42 chromosomes in females, all acrocentric, and 2n=41 chromosomes in males, with 40 acrocentric and 1 medium-sized metacentric chromosome. These differences concern with a multiple system of sex chromosome determination $X_1X_1X_2X_2/X_1X_2Y$ (FN=42) in *B. gauderio*. The analysis of nucleolar organizer regions by Ag-NOR and FISH 18S banding revealed a simple NOR system in *A.* prope *albifrons* and *R. hahni* and a multiple NOR system in *B. gauderio*, that is unusual for Gymnotiformes fishes. Constitutive heterochromatin was mainly found in the pericentromere region in most of the chromosomes of the three species, although each species had its own peculiarities. The B chromosomes in *Apteronotus* prope *albifrons* demonstrated heterochromatin positioned in the centromeric and telomeric regions whereas *R. hahni* presented conspicuous blocks of heterochromatin on the long arms in three submetacentric pairs.

Brachyhypopomus gauderio showed blocks of heterochromatin on the long arm in the interstitial and telomere positions. The finding of B chromosomes in *A.* prope *albifrons* represents the first description of these elements in the Gymnotiformes order. Although the karyotype of this species is similar with that described for populations in the Amazon basin, the presence of B chromosomes could represent a specific characteristic of this population. A comparative analysis of karyotypes of *R. hahni* with other species of the genus showed a relatively conservative structure suggesting 2n=50 as a common number in this group. The karyotype of *B. gauderio*, a new species, provides an important reference for future chromosome studies of the *Brachyhypopmus* Mago-Lecia, 1994, and it might be also significant for cytotaxonomy in this group. The cytogenetic data also demonstrate the need of more comparative cytogenetic studies in the families of the highly diversified and taxonomically difficult complex Gymnotiformes.

Keywords

fish cytogenetics**,** B chromosomes, C-banding, ribosomal DNA, sex chromosomes

Introduction

The order Gymnotiformes is a group with high species diversity and about 179 species have been listed so far (Crampton 2011).

Karyotype diversity is well known in Gymnotiformes, especially in the genera *Gymnotus* Linnaeus, 1758 and *Eigenmannia* Jordan et Evermann, 1896. Regarding this order, the diploid number ranges from 2n = 22 or 24 in *Apteronotus albifrons* Linnaeus, 1766 (Hinegardner and Rosen 1972, Almeida-Toledo et al. 1981, respectively) to 2n=54, the highest diploid number recorded in *Gymnotus carapo* Linnaeus, 1758 and *Gymnotus mamiraua* Albert et Crampton, 2001 (Foresti et al. 1984, Milhomem et al. 2007, respectively).

The karyotype variability in Gymnotiformes also involves simple and multiple sex chromosome systems such as those registered in Gymnotidae, Hypopomidae and Sternopygidae species. The sex chromosome systems XX/XY and ZZ/ZW were described in *Eigenmannia virescens* Valenciennes, 1836 (Almeida-Toledo et al. 2001, Silva et al. 2009) and $X_1X_1X_2X_2/X_1X_2Y$ in *Eigenmannia* sp, (Almeida-Toledo et al. 2000a), *Gymnotus* sp. (Silva and Margarido 2005) and *Brachyhypopomus gauderio* (earlier misidentified as *B. pinnicaudatus* Hopkins, 1991 (Almeida-Toledo et al. 2000b). Cytogenetic information on the Apteronotidae, Hypopomidae and Rhamphichthyidae families is very scarce in the literature. Table 1 shows the cytogenetic data reported for these families to date.

In spite of extensive karyotype variability, a review of the cytogenetic studies of Neotropical freshwater fish shows that, to date, there is no record of B chromosomes (or supernumerary chromosomes) in Gymnotiformes (Carvalho et al. 2008, Oliveira et al. 2009). These chromosomes are not essential to cell functioning and may be derived from autosomes and sex chromosomes in intra- and interspecies crosses (Camacho et al. 2000). The B chromosomes are well documented in fishes and have been reported in more than 60 species. Characiformes appear to have a high proportion of species with B chromosomes: 50.8% of a total of 61 species examined (Carvalho et al. 2008). Other groups show a lower prevalence of species with B chromosomes; for

Table 1. Cytogenetic data available for Apteronotidae, Rhamphichthyidae and Hypopomidae families. FN: fundamental number; m: metacentric; sm: submetacentric; st: subtelocentric; a: acrocentric; AM: Amazonas; PR: Paraná; SP: São Paulo; PA:Pará.

Family/species	Locality	2n	FN	Karyotype	Sex chromo-somes	Reference
Apteronotidae						
Apteronotus albifrons	Amazon	24	42	14m+2sm+2st+6a		Howell (1972)
A. albifrons	Marajó Island, PA	24	42	12m+4sm+2st+6a		Almeida-Toledo et al. (1981)
A. prope *albifrons*	Upper PR, PR	24	42	14m+2sm+2st+6a		Present study
A. albifrons		22				Hinegardner and Rosen (1972)
Apteronotus sp.	São Paulo	52	98	46m/sm+ 6st/a		Almeida-Toledo et al. (2007)
Parapteronotus bonaparti (*A. anas*)	Manaus (AM)	52	94	30m+12sm+10a		Almeida-Toledo et al. (2007)
P. hasemani (*Apteronotus*)	Manaus, AM	52	94	26m+16sm+10a		Almeida-Toledo et al. (2007)
Rhamphichthyidae						
Rhamphichthys hani	Upper PR, PR	50	94	20m+24sm+6a		Present study
R. prope *pantherinus*	Amazon	52	100	38m+10sm+4st		Almeida-Toledo (1978)
R. marmoratus	Amazon	50	94	44m/sm+6st/a		Silva (2010)
R. rostratus	Amazon	50	92	42m/sm+8a		Silva (2010)
Hypopomidae						
Brachyhypopomus brevirostris	Amazon	36	42	4m+2sm+8st+22a		Almeida-Toledo (1978)
B. gauderio	Upper PR, PR	♀ 42	42	42a	$X_1X_1X_2X_2$	Present study
		♂ 41	42	1m+40a	X_1X_2Y	
	Tietê River, SP	♀ 42	42	42a	$X_1X_1X_2X_2$	Almeida-Toledo et al. (2000b)
		♂ 41	42	1m+40a	X_1X_2Y	(as *B. pinnicaudatus*)
Hypopomus artedi	Amazon	38	70	32m/sm+6st/a		Almeida-Toledo (1978)
Hypopygus lepturus	Amazon	50	96	16m+20sm+10st+4a		Almeida-Toledo (1978)

example, 34.42% in Siluriformes and 8.19% in Perciformes, and other orders, such as Beloniformes, Cyprinodontiformes and Synbranchiformes, have only one species with B chromosomes (1.63% per order) (Carvalho et al. 2008).

Although Neotropical fishes show considerable variability regarding the number of B chromosomes (1–16), usually 1–4 chromosomes are present. The B chromosomes show wide variations in size, from very small (micro), as in *Moenkhausia sanctaefilomenae* Steindachner, 1907 (Foresti et al. 1989, Portela-Castro et al. 2001) and *Rineloricaria pentamaculata* Langeani et Araujo, 1994 (Errero-Porto 2010), small, as in *Cyphocharax modestus* Fernández-Yépez, 1948 (Vênere et al. 1999), medium sized, as in *Rhamdia quelen* Quoy et Gaimard, 1824 and *Rhamdia branneri* Haseman, 1911 (Fenocchio and Bertollo 1990, Abucarma and Martins-Santos 2001, respectively) and large, such as the different species of the genus *Astyanax* Baird et Girard, 1854 (Moreira-Filho et al. 2004).

Methodologies such as C-banding have revealed the nature of heterochromatic B chromosomes with repetitive DNA sequences (Camacho et al. 2000). Totally heterochromatic B chromosomes constitute a common situation in many species of Neotropical fishes; however, they can be completely euchromatic, as in *Moenkausia sanctefilomenae* (Foresti et al. 1989), *Steindachnerina insculpta* Fernández-Yépez, 1948 (Oliveira and Foresti 1993), *Characidium* prope *zebra* (Vênere et al. 1999) and *Rhamdia quelen* (Moraes et al. 2009), or partially heterochromatic, as in some populations of "*Astyanax scabripinnis* complex" (Moreira-Filho et al. 2004), *Rhamdia hilarii* Valenciennes, 1840 (Fenocchio and Bertollo 1990) and *Rhamdia quelen* (Moraes et al. 2009).

Material and methods

We analysed 51 specimens (24 males and 27 females) of *Brachyhypopomus gauderio*, 6 specimens of *Apteronotus* prope *albifrons* (4 males and 2 females) and 19 specimens of *Rhamphichthys hahnii* (8 males, 7 females and 4 undetermined sex). The specimens were collected in rivers (Baía and Ivinhema) and lagoons of the upper Paraná River floodplain near the town of Porto Rico (PR), Brazil (Fig. 1). Voucher specimens were deposited in the fish collection of the Research Nucleus in Limnology, Ichthyology and Aquaculture (Nupélia), Universidade Estadual de Maringá, PR Brazil, as *Apteronotus* prope *albifrons* (NUP9621), *Rhamphichthys hahni* (NUP9623) and *Brachyhypopomus gauderio*, (earlier misidentified as *Brachyhypopomus* prope *pinnicaudatus,* NUP9622). *B. gauderio* represents a new species of the southern Brazil, Uruguay and Paraguay described by Giora and Malabarba (2009). Fig. 2 shows photographs of each species.

Conventional staining

We obtained metaphase chromosomes from kidney cells using the air-drying technique proposed by Bertollo et al. (1978) and stained with 5% Giemsa in phosphate

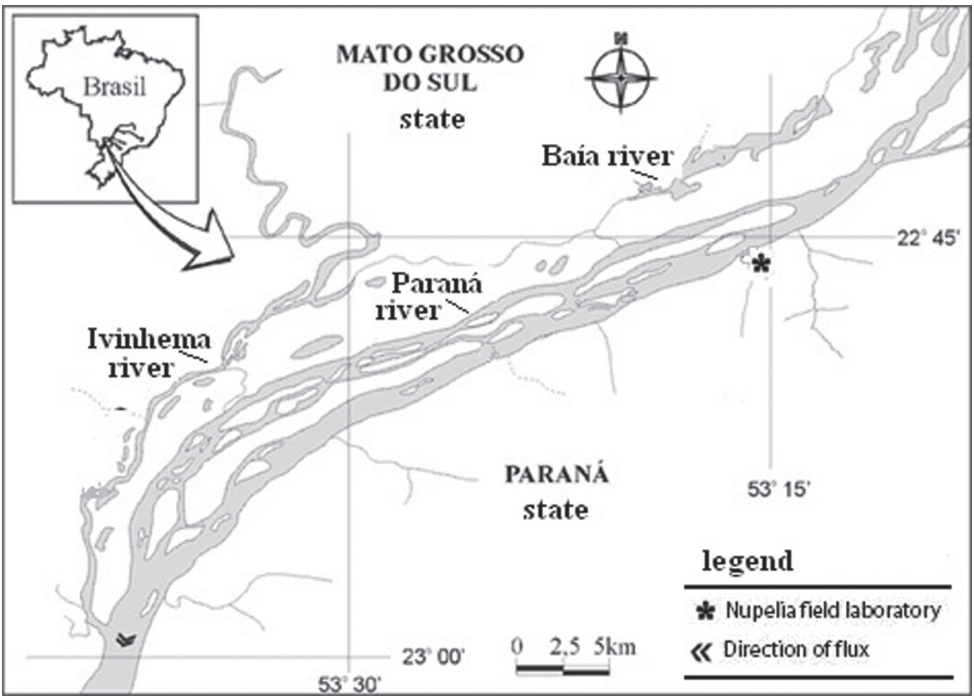

Figure 1. Collections sites of studied species**.** Map of Brazil showing the localization of Paraná river, in the Paraná state. Hhydrographic map of the floodplain of the Upper Paraná River and its tributaries, Baía and Ivinhema Rivers.

buffer (pH 6.8). We used benzocaine solution to anaesthetize the fish before sacrificing them. We used the arm ratio criteria (AR) to characterize chromosome morphology, as suggested by Levan et al. (1964): metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a) chromosomes.

Chromosome banding

We used the C-banding method and staining with Giemsa after treatments with 0.1M HCl, $Ba(OH)_2$ and 2×SSC solutions to analyse the distribution of heterochromatin, as described by Sumner (1972), and we used the silver nitrate staining method (Ag-NOR) to identify the nucleolus organizer regions (NORs), as described by Howell and Black (1980).

Fluorescent in-situ hybridization (FISH)

The probes used to detect 18S rDNA in the FISH analyses were obtained by amplified and cloned fragments of *Oreochromis niloticus* Linnaeus, 1758 (kindly provided

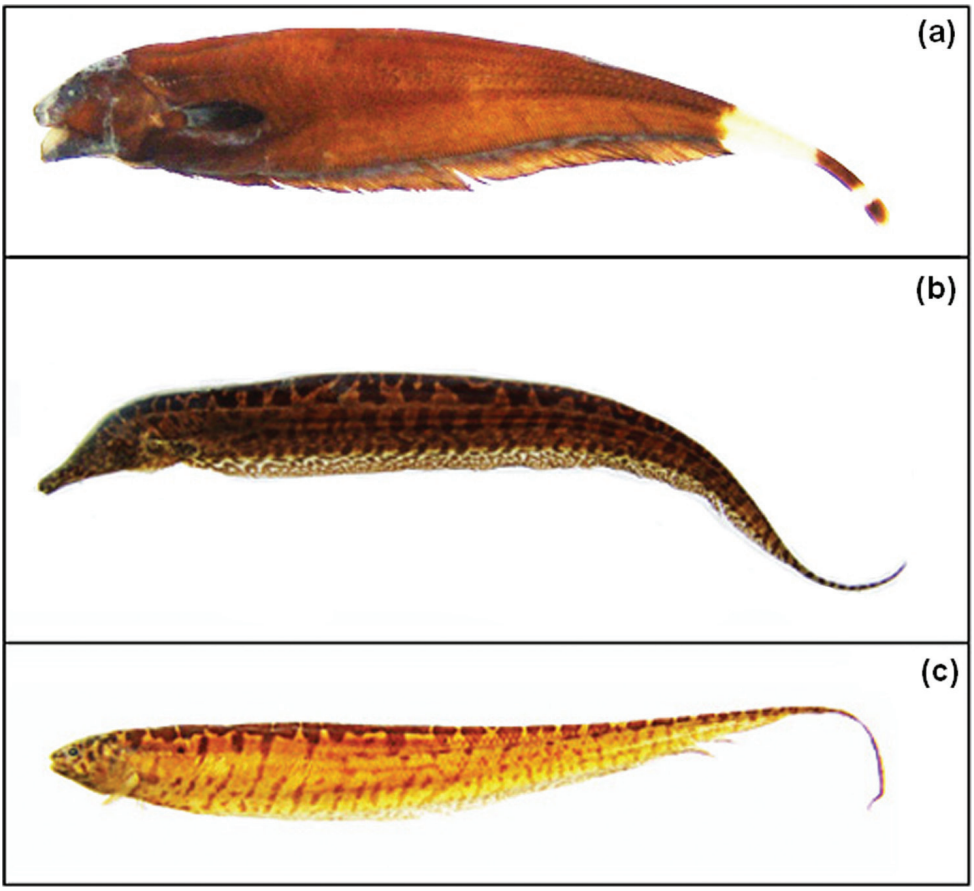

Figure 2 a–c. Specimens of: **a** *Apteronotus* prope *albifrons* (102,7 mm SL, NUP9621) **b** *Rhamphichthys hahni* (244,4 mm SL, NUP9623) and **c** *Brachyhypopomus gauderio* (140,9mm SL, NUP9622).

by Dr Cesar Martins of the Universidade Estadual Paulista, Botucatu SP Brazil). We used the methods of Heslop-Harrison et al. (1991) and Cuadrado and Jouve (1994) to perform the FISH analyses, with modifications by Swarça et al. (2001). The probes were labelled with biotin 14-dATP via nick translation (Bio Nick Labeling System - Gibco, BRL). We incubated the slides with RNase (37 ºC, 1 h) and then treated them with 30 µl of hybridization mixture containing 100–300 ng of labelled probes (3 µl), 15 µl 100% formamide, 20×SSC (0.5 µl), 0.1 µl calf thymus DNA, 0.6 µl 50% Dextran and 0.1 µl 10% SDS. We denatured the material at 90 °C for 10 min and carried out hybridization overnight at 37 °C in a humidified chamber. All post-hybridization washes were carried out in 2×SSC, 20% formamide in 0.1×SSC, 0.1×SSC and 4×SSC/0.2% Tween 20, at 42 °C. We detected the probes using a solution of 5% BSA and FITC-conjugated avidin. We then counterstained the chromosomes using 30 µl of 0.2% propidium iodide and mounted the slides in Vectashield antifade (Vector).

Results

Apteronotus prope *albifrons* showed a diploid number of 24 chromosomes and a karyotype formula of 14m+2sm+2st+6a with a fundamental number (FN) of 42 (Fig. 3a). We observed constitutive heterochromatin distributed in small blocks throughout the pericentromere regions of most of the chromosomes (Fig. 3b) and in conspicuous blocks in the region adjacent to the nucleolar pair 4 secondary constriction (Fig. 3b). The Ag-NOR and 18S rDNA sites were located on the short arm of chromosome pair 4 (Fig. 4a, b), coinciding with the secondary constriction evident in some metaphases.

In addition to the normal chromosome complement, we found that three specimens of *Apteronotus* prope *albifrons* had one to three B microchromosomes in their somatic cells, with inter- and intra-individual variations (Fig. 3a, box and Fig. 5a–c). The B chromosomes showed no homology with the other chromosomes of the complement and, morphologically, these chromosomes were classified as acrocentric. We observed constitutive heterochromatin in the pericentromere and terminal position of the extra chromosomes (Fig. 5d).

Rhamphichthys hahni showed a diploid number of 50 chromosomes for both sexes and a karyotype formula of 20m+24sm+6a with a fundamental number (FN) of 94 (Fig. 3c). We observed constitutive heterochromatin in the pericentromere regions of most of the chromosomes and conspicuous blocks in three submetacentric chromosomes of the complement and also in the position close to secondary constriction of pair 7 (Fig. 3d). The Ag-NORs and 18S rDNA sites were located in the short arm of chromosome pair 7, coincideing with a terminal secondary constriction (Fig. 3c, box, and Fig. 4 c,d). We also observed an NOR region-sized heteromorphism in this pair (Fig. 4c, d).

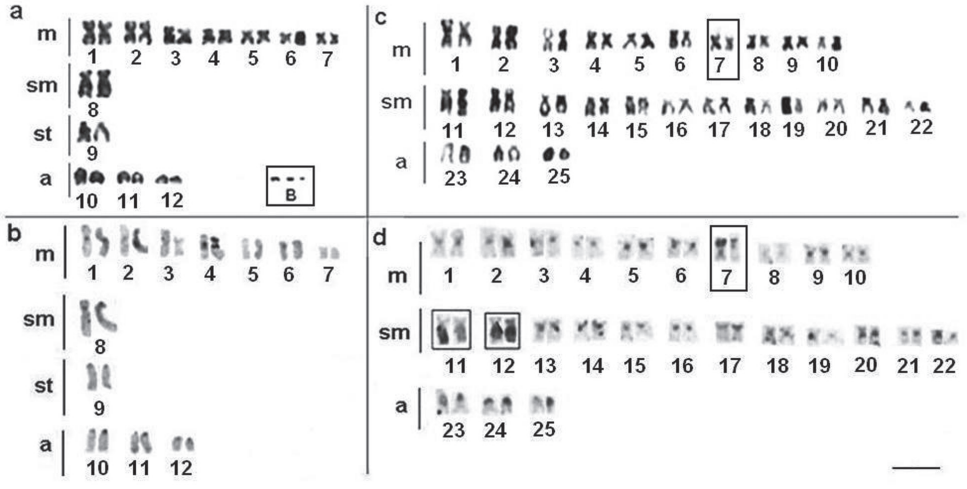

Figure 3 a–d. Karyotypes of *Apteronotus* prope *albifrons* and *Rhamphichthys hahni* after: Giemsa-staining **a**, **c** respectively and C-banding **b**, **d**; In evidence, B chromosomes of *A.* prope *albifrons* **a** and heterochromatic blocks adjacent to the nucleolar regions 4 **b**; **c** terminal secondary constriction on pair 7 and conspicuous heterochromatic regions on pairs 11 and 12 **d** in the karyotype of *R. hahni*. Bar = 5µm.

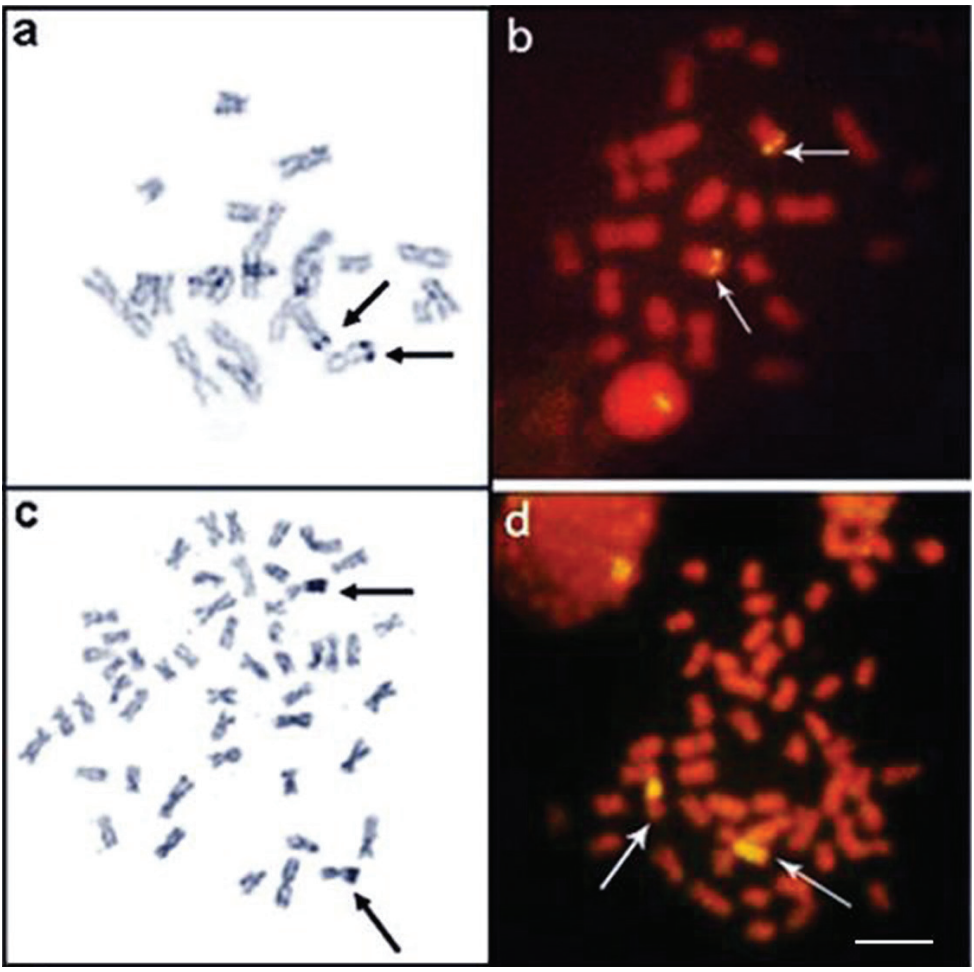

Figure 4 a–d. Metaphases showing Ag-NORs-bearing chromosomes and FISH using 18S rDNA probe of: *Apteronotus* prope *albifrons* **a**, **b** and *Rhamphichthys hahni* **c**, **d** Note an evident NOR region-sized heteromorphism of pair 7 of *R. hahni* **c**, **d**. Bar = 5µm.

Brachyhypopomus gauderio presents 2n=42 chromosomes in females and 2n=41 chromosomes in males. The female karyotype showed acrocentric chromosomes only (Fig. 6a) and the male karyotype showed 40 acrocentric chromosomes and one medium-sized metacentric chromosome (Fig. 6b). We found that the fundamental number for both sexes is 42. The difference in karyotype structure between the sexes demonstrates the presence of sex chromosomes, with a metacentric chromosome corresponding to a Y chromosome. Chromosomal pairs 11 and 14 are X_1 and X_2 in males and females, respectively. In fact, this condition characterizes a system of multiple sex chromosomes of the type $X_1X_1X_2X_2/X_1X_2Y$.

We found C-positive blocks (constitutive heterochromatin) in the pericentromere regions of all chromosomes in males and females (Figure 6c) of *B. gauderio*, including

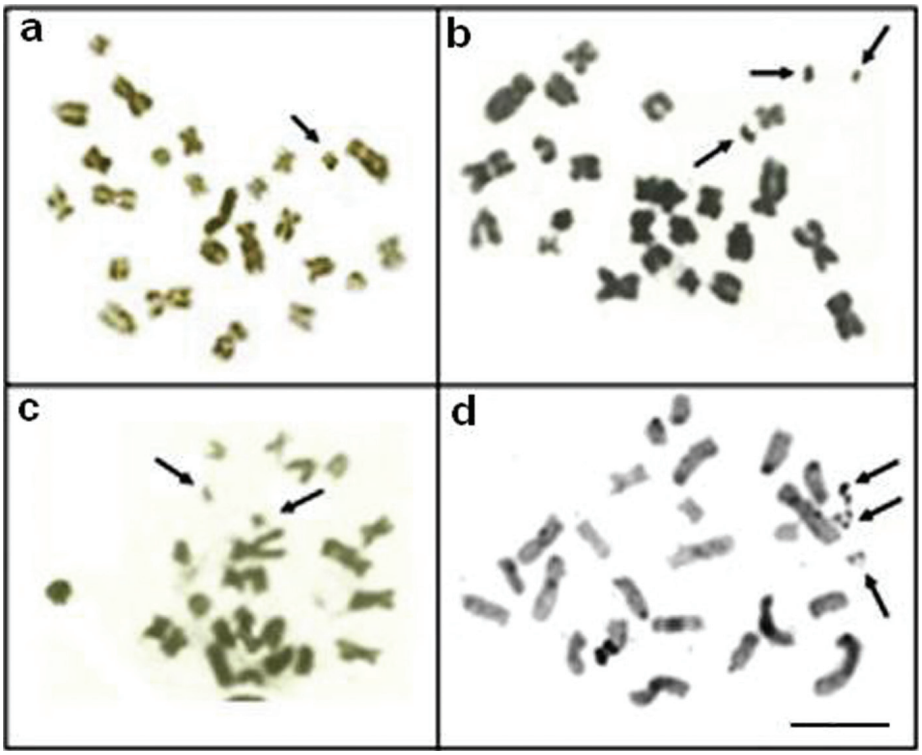

Figure 5 a–d. Somatic metaphases of *Apteronotus* prope *albifrons* stained with Giemsa **a**,**b** and **c** and C-banded **d** showing the B chromosomes (arrows). Bar = 5 μm.

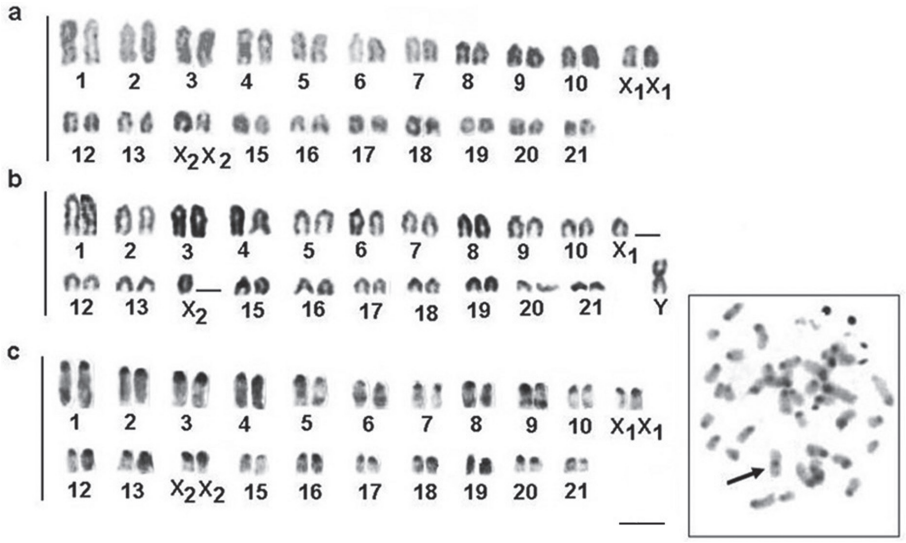

Figure 6 a–c. Karyotypes and metaphases of *Brachyhypopomus gauderio* after: Giemsa- staining in female **a** male **b** and C-banding in female **c**. Right, C-banded metaphase showing the Y chromosome (arrow). Bar = 5μm.

the pericentromere region of the Y metacentric chromosome (Fig. 6, in the boxes). Furthermore, some chromosomes showed blocks of heterochomatin in the long arm in the interstitial and telomere positions. We observed Ag-NOR sites in the short and long arms of the acrocentric pairs (Fig. 7a, b), and the FISH technique revealed eight fluorescent signals (rDNA sites), including the sites stained by silver (Figure 7c). Figure 6d shows a correlation between some of the Ag-NOR and 18S rDNA sites.

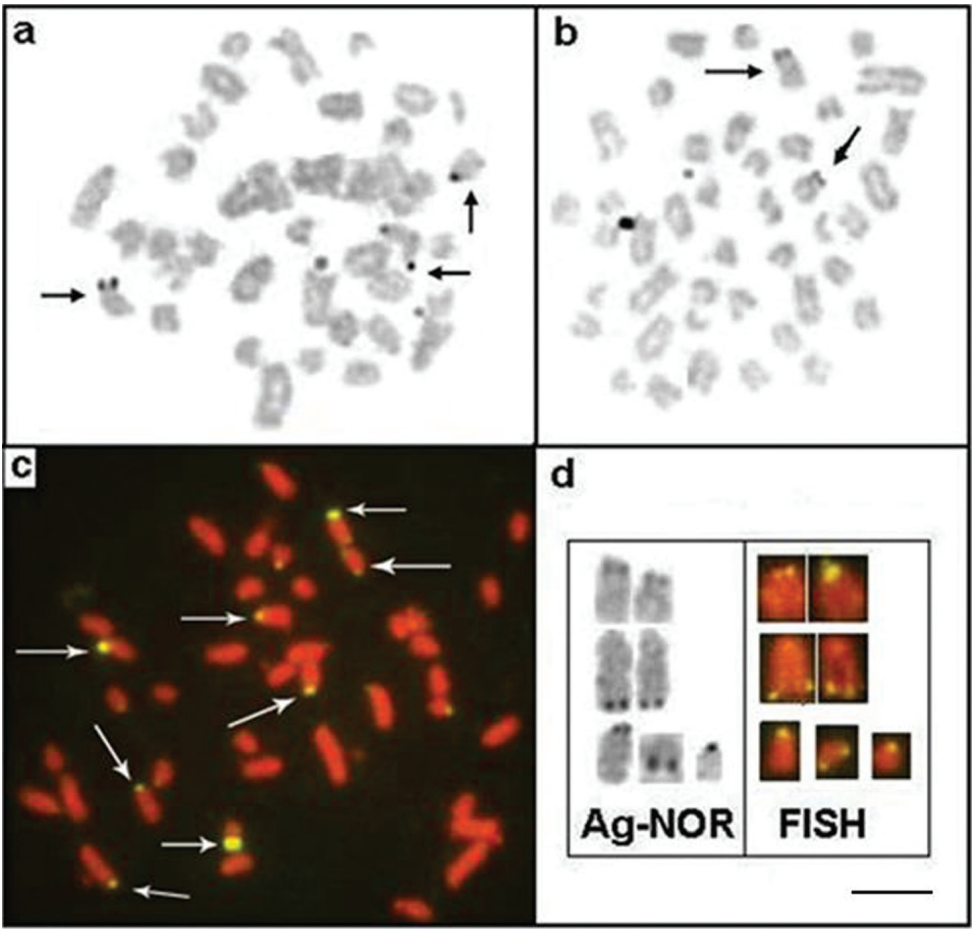

Figure 7 a–d. Metaphases of *Brachyhypopomus gauderio* showing NORs regions: Ag-NORs-bearing chromosomes in the male **a** female **b** and eight 18S rDNA sites, arrows **c**; **d** correlation between some Ag-NOR and 18S rDNA regions. Bar = 5µm.

Discussion

The karyotype structure we describe herein for *Apteronotus* prope *albifrons* (2n=24, 14m+2sm+2st+6a) is similar to the one reported by Howell (1972) from the Amazon basin, but differs from that of a population studied by Almeida-Toledo et al. (1981)

from Marajó Island, Pará) (Table 1). These small variations between the chromosome formula described for *A. albifrons* are expected as a result of different interpretations of the classification of chromosomes when the existence of a secondary constriction in pair 4 is taken into account. Thus, we concluded that the karyotypes of *A.* prope *albifrons* (in the current study) and *A. albifrons* (Amazon basin) are similar, except for the presence of B chromosomes in the population inhabited the Parana River. According to Santana (2003), *A. albifrons* is most likely a complex of closely related cryptic species. If this is applicable to the *A.* prope *albifrons* populations discussed here, the presence of B chromosomes could be a karyotaxonomic character. The occurrence of B chromosomes in *A.* prope *albifrons* only could represent a specific characteristic of the population (probably, a geographical variation) in the upper Parana River floodplain.

Our report of B chromosomes in *A.* prope *albifrons* is the first description of these elements in the Gymnotiformes order. The mitotic instability presented by the B chromosomes of *A.* prope *albifrons* is likely due to their non-Mendelian behaviour during cell division, which is a common feature attributed to B chromosomes in other species.

The origin of B chromosomes is not yet well understood, although intraspecific origin of B chromosomes in some Neotropical fishes have been suggested as a result of the non-disjunction of an autosome, the formation of isochromosomes, centric fragments resulting from chromosomal rearrangements and amplifications of paracentric regions of a fragmented A chromosome (Camacho 2000, Carvalho et al. 2008).

Heterochromatinization plays an important role in the differentiation of B chromosomes. The B chromosomes are thought to be composed of repetitive sequences (heterochromatin) that lack protein-coding genes. The fact that the B chromosomes of *A.* prope *albifrons* appeared to be partially heterochromatic does not provide evidence of the presence or absence of coding genes. This would require a detailed molecular cytogenetic analysis with specific probes for FISH. Regarding the pattern of C bands of the other chromosomes of *A.* prope *albifrons*, we noted a similarity in the location of the heterochromatin when comparing it with the population studied by Almeida-Toledo et al. (1981).

The presence of only one NOR-bearing chromosome pair in *A.* prope *albifrons* in this study (confirmed by FISH) corresponds to the condition found in an Amazonian basin population of *A. albifrons* analysed by Almeida-Toledo et al. (1981). The presence of a nucleolar pair in most species of Gymnotiformes is a common pattern (see the review of Arai 2011). We found that the secondary constriction in this pair (no. 4) had a negative C-band pattern. This was also reported by Almeida-Toledo et al. (1981) for this species and for *Electrophorus electricus* Linnaeus, 1766 (Gymnotidae from the Amazon River (Fonteles et al. 2008).

Finally, we analysed the karyotypic data available so far for the Apteronotidae family and observed a relative numerical and structural variability, ranging from 2n=22 and 24 to 52 (Table 1), suggesting an evolutionary history of chromosomal rearrangements. This trend can also be seen when comparing the karyotypic formulas of the *Apteronotus* La Cépede, 1800 and *Parapteronotus* Albert, 2001 genera (Table 1). However, the absence of cytogenetic data for other species of the Apteronotidae family makes it difficult to understand the karyotypic interrelationships and the types of rear-

rangements that have resulted in the diploid values in this group so far. In addition, cytogenetic information for other species of this family would be helpful for clarifying the origin of the B chromosomes discussed herein.

The karyotype structure of *Rhamphichthys hani* and *Brachyhypopomus gauderio*

Cytogenetic studies in the Rhamphichthyidae family are still scarce and the karyotype of *R. hahni* is reported herein for the first time. The cytogenetic data available for *Rhamphichthysi* Müller et Troschel, 1848 show less variation in diploid number and chromosome structure (see Table 1). For example, the comparison of the karyotype of *Rhamphichthys rostratus* Linnaeus, 1766 (Silva 2010) with that of *R. hahni* (2n=50, 20m+24sm+6a) in the current study shows that their karyotype formula are similar. Although these data reflect a relatively more conservative chromosomal evolution in the Rhamphichthyidae family, the diploid numbers of 50–52 chromosomes do not exclude the possibility of rearrangements such as fissions/fusions occurring during the karyotypic evolution of this group, besides those associated with changes in the karyotype formulae.

The presence of NOR in only one pair of chromosomes found in *R. hahni* coincides with the patterns observed in *Rhamphichthys marmoratus* Castelnau, 1855 and *Rhamphichthys rostratus* (Silva 2010). The NOR-sized heteromorphism found in the nucleolar pair (no. 7) in *R. hahni* suggests a structural variation in the number of DNAr cistrons among the homologues. This phenomenon could have originated from an unequal crossing-over, as found in many fish species. For example, among the Gymnotiformes, NOR-sized heteromorphisms were visualized in species of *Eigenmannia* (Foresti et al. 1981, Almeida-Toledo et al. 1996, Foresti 1987) and *Steatogenys* Boulenger, 1898 genera (Cardoso et al. 2011).

The C-band pattern in *R. hahni* is similar to the heterochromatin location in the pericentromere region in many species of Gymnotiformes. The NOR region is associated with heterochromatin, which is frequently found in many fish species. However, three medium-sized submetacentric chromosomes show conspicuous heterochromatic blocks on the long arm. The distribution and amount of heterochromatin may have an important evolutionary role in the chromosomes of many fish species, including sex chromosomes, as reported in *Eigenmannia virescens* (Almeida-Toledo et al. 2001, Silva et al. 2009) and *Steatogenys elegans* Steindachner, 1880 (Cardoso et al. 2011). We found no evidence of sex chromosome differentiation in *R. hahni*, and the heterochromatic blocks on the long arm of the metacentric chromosomes could be useful markers of this species. However, other species have to be analysed.

The chromosome formula discovered by us in *B. gauderio* corresponds to the data previously reported by Almeida-Toledo et al. (2000b) (see Table 1). There is little cytogenetic information available on the *Brachhypopomus* genus. In addition to the above mentioned species, the karyotype of *Brachhypopomus*. *brevirostris* Steindachner, 1868

from the Amazon basin (Almeida-Toledo 1978) was also recorded karyotype formula; the sex chromosomes have still not been distinguished.

The origin of the multiple sex determination system, $X_1X_1X_2X_2/X_1X_2Y$ in *B. gauderio* (cited as *B. pinnicaudatus*) was discussed by Almeida-Toledo et al. (2000b) using C-band, DAPI staining and FISH (with a telomere probe) techniques. Based on these analyses, the authors suggested that the Y chromosome originated from a centric fusion (Robertsonian fusions) involving two average sized acrocentric chromosomes. In the present study, *B. gauderio* showed a C-band pattern similar to that detected in the population from the Tietê River (Almeida-Toledo et al. 2000b), including the C-positive blocks on the pericentromere region of the Y metacentric chromosome, strengthening the hypothesis discussed above.

The detection of up to eight chromosomes with a fluorescent signal (18S rDNA sites) indicates multiple NOR systems in *B. gauderio*. Furthermore, the difference in the results between the methodologies (Ag-NOR and FISH) suggests that not all ribosomal DNA sites were active in the previous interphase. Also, chromosomal rearrangements such as translocations and/or transpositions, resulting in the dispersion of ribosomal genes, might explain this variability. No information is provided on the sites of NORs in the *B. pinnicaudatus* (currently *B. gauderio*) population analysed by Almeida et al. (2000b); however, in some individuals from an *Eigenmania* sp.1 population Almeida-Toledo et al. (1996) found four chromosomes with NOR regions.

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Cytotaxonomy of the subgenus *Artibeus* (Phyllostomidae, Chiroptera) by characterization of species-specific markers

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Abstract

The genus *Artibeus* represents a highly diverse group of bats from the Neotropical region, with four large species occurring in Brazil. In this paper, a comparative cytogenetic study was carried out on the species *Artibeus obscurus* Schinz, 1821*, A. fimbriatus* Gray, 1838*, A. lituratus* Olfers, 1818 and *A. planirostris* Spix, 1823 that live sympatrically in the northeast of Brazil, through C-banding, silver staining and DNA-specific fluorochromes (CMA_3 and DAPI). All the species had karyotypes with 2n=30,XX and 2n=31,XY_1Y_2, and FN=56. C-banding showed constitutive heterochromatin (CH) blocks in the pericentromeric regions of all the chromosomes and small CH blocks at the terminal region of pairs 5, 6, and 7 for all species. Notably, our C-banding data revealed species-specific autosomic CH blocks for each taxon, as well as different heterochromatic constitution of Y_2 chromosomes of *A. planirostris*. Ag-NORs were observed in the short arms of chromosomes 5, 6 and 7 in all species. The sequential staining $AgNO_3$/CMA_3/DA/DAPI indicated a positive association of CH with Ag-NORs and positive CMA_3 signals, thus reflecting GC-richness in these regions in *A. obscurus* and *A. fimbriatus*. In this work it was possible to identify interespecific divergences in the Brazilian large *Artibeus* species using C-banding it was possible provided a suitable tool in the cytotaxonomic differentiation of this genus.

Keywords
C-banding, Ag-NOR, CMA_3/DA/DAPI, Cytotaxonomy

Introduction

The genus *Artibeus* Leach, 1821 has been divided into two main groups based on body size. The species with larger body size have been classified as subgenus *Artibeus* and the species with smaller body size as the subgenus *Dermanura* Gervais, 1856. In addition, a new subgenus *Koopmania* Owen, 1991 was proposed by Owen (1987, 1991) to set apart one of the species, *A. concolor* Peters, 1865. However, this subgenus was later disregarded by Van De Bussche et al. (1998) based on morphological, enzymatic, molecular and karyotypic analysis. The taxonomic classification proposed by Hoofer et al. (2008) recognized *Artibeus* as a distinct subgenus from *Dermanura*. Recently, Marchán-Rivadeneira et al. (2010) described the genus *Artibeus* as constituted by 11 large-body size species including *A. concolor* as the basal taxon. Its distributional range is restricted to the Neotropical region. The genus is widely distributed from Mexico to northern Argentina, including the Antillean islands in the Caribbean (Simmons 2005).

The extensive similarity of morphometric characters, high degree of shape diversity and overlapping of natural habitats have hindered accurate identification of the large *Artibeus* along their distribution, particularly in the Neotropical region (Haynes and Lee 2004, Hollis 2005). A typical example is the northeastern region of Brazil where four species of the large *Artibeus* (*A. obscurus* Schinz, 1821, *A. fimbriatus* Gray, 1838, *A. lituratus* Olfers, 1818 and *A. planirostris* Spix, 1823) were formally recorded living in sympatry (Taddei et al. 1998, Araújo and Langguth 2010). In this region, similarity in morphometric measurements (e.g. cranial distances and external dimensions) and geographical variation of *A. planirostris* are main reasons for confusing taxonomy (Guerrero et al. 2003, Simmons 2005).

Since the systematic classification of subgenus *Artibeus* remains subject of several discussions concerning phylogenetic relationships and actual taxonomic status of species, the use of complementary information may help to define species more precisely (Larsen et al. 2010). For other Mammalian groups, such as primates, felines and rodents, classical and molecular cytogenetic analysis have been successfully allied to taxonomic studies to identify species since chromosomes are not affected by adaptation process to different feeding niches as cranial and general gross anatomies (Granjon and Dobigny 2003, Garcia and Pessoa 2010).

In this work a karyotypic characterization of *A. obscurus* Schinz, 1821, *A. fimbriatus* Gray, 1838, *A. lituratus* Olfers, 1818 and *A. planirostris* Spix, 1823 from northeastern of Brazil was performed by the cytogenetic techniques – conventional analysis, C-banding, Ag-NOR and triple staining CMA_3/DA/DAPI. The data were helpful to carry a comparative analysis of those species, in terms of interspecific differences, and also to provide a better identification of them.

Material and methods

Based on literature (Handley 1989, Taddei et al. 1998), the following characters were used to diagnose the species: presence of fur on the forearms, structure of legs and interfemoral membrane; color of body, dorsal and ventral fur; facial stripes; form of nose leaf and its relationship with the upper lip; shape of the pre- and postorbital process and postorbital constriction and the presence or absence of the 3rd molar. The identification process also included the following 10 measurements: length of forearm, condylobasal length; length of maxillary tooth-row; length of lower tooth-row; length of mandible; breadth across upper canines; mastoidal breadth; zygomatic breadth; postorbital constriction; breadth across upper molars.

After identification, cytogenetic studies were carried out on 53 *Artibeus* specimens from the state of Pernambuco, northeastern Brazil. Voucher specimens are deposited in the Mammalian collection at the Department of Systematic and Ecology, Federal University of Paraíba, João Pessoa, Paraiba, Brazil. The specimens studied were six males and eight females of *Artibeus obscurus*; two males and four females of *A. fimbriatus*; eight males and five females of *A. planirostris*; ten males and ten females of *A. lituratus* captured at different sites across the Pernambuco State: Igarassu (07°50'02"S, 34°54'21"W), Água Preta (08°42'27"S, 35°31'50"W), Rio Formoso (08°39'50"S, 35°09'32"W), Ipojuca (08°24'00"S, 35°03'45"W) and Recife (08°03'14"S, 34°52'51"W) (see also Appendix).

Metaphase spreads were obtained from bone marrow cells according to conventional procedures and staining with Giemsa. C-banding and silver staining were performed according to Sumner (1972) and Howell and Black (1980), respectively. Triple staining CMA_3/DA/DAPI was carried out according to Santos and Souza (1998a).

For sequential staining ($AgNO_3$/CMA_3/DA/DAPI), the slides stained by silver nitrate were distained after photographing (Dos Santos Guerra 1991) and re-stained by CMA_3/DA/DAPI. Photomicrographs were taken using Leica DMLB photomicroscope for C-banding and silver staining. Sequential staining images were captured by IM50 capture system.

Results

All four species shared the same diploid number (2n=30, gap XX; 2n=31, gap XY_1Y_2) and fundamental number FN=56. Chromosomes were meta-submetacentric (1-4, 8-14), subtelocentric (5, 6, 7 and X) and two small acrocentric (Y_1 and Y_2). Except for the size of Y_1 and Y_2 chromosomes, it was not found any intraspecific variation between species analyzed with conventional staining.

C-banding revealed constitutive heterochromatin (CH) in the pericentromeric region of all the autosomes and small heterochromatic blocks were observed in the terminal region of chromosome pairs 5, 6 and 7 (Fig. 1a–d). The karyotype of *A. obscurus* (Fig. 1a) exhibited interstitial blocks in the short and long arms of pair 1, as well as in the long arms of pairs 2, 5, 6 and in the terminal region of the short arm of pair 9. The *A. planirostris* karyotype

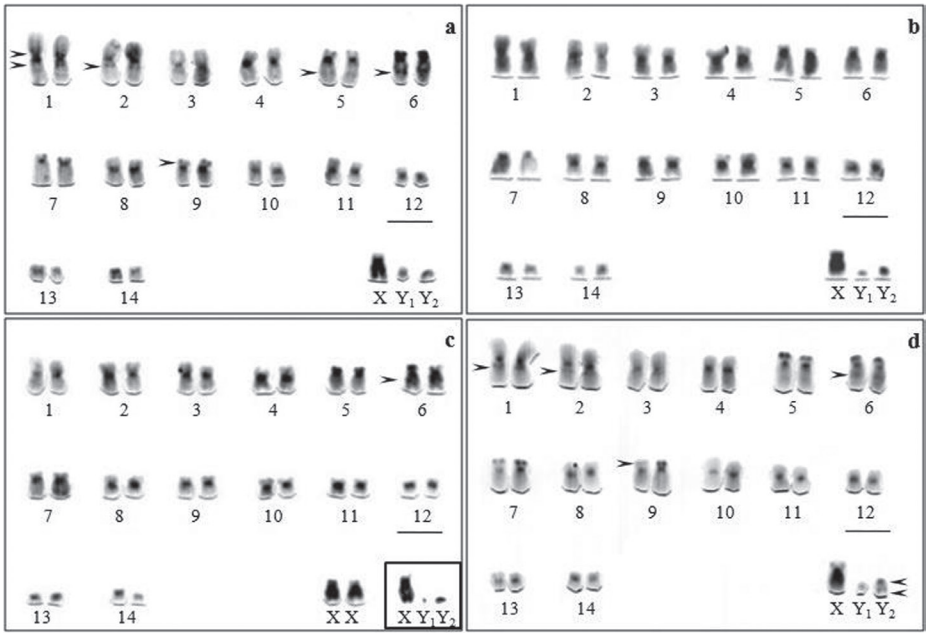

Figure 1. C-banding of *A. obscurus* **(a)** *A. fimbriatus* **(b)** *A. lituratus* **(c)** and *A. planirostris* **(d)** karyotypes. The arrowheads indicate a particular set of CH blocks in each species. Bar = 5 μm.

(Fig. 1d) has the same CH pattern but lacks interstitial blocks in the short arm of chromosome 1. Absence of an interstitial block on the chromosome 6 distinguished karyotype of *A. fimbriatus* from the other investigated karyotypes (Fig. 1). In all the material examined the long arms of the X chromosomes were more darkly stained when compared with the euchromatin of the autosomes. The Y_2 appeared almost entirely heterochromatic in all species, except for *A. planirostris* which showed pericentromeric and distal blocks (Fig. 1d). The pattern of the Y_1 could not be determined with precision due to its punctiform size.

Table 1 shows exhibits the C-banding pattern in chromosomal complement in all species analyzed.

Silver staining (Ag-NORs) showed three pairs of NORs in the terminal region of the short arms in chromosomes 5, 6 and 7 in all species. As a result of remarkable variation in expression and activity, Ag-NORs were counted up to 100 nuclei, which were randomly selected, and the mean number of Ag-NORs per nucleus was determined for each case (Table 2).

The sequential staining $AgNO_3$/CMA_3/DA/DAPI showed a correlation between CMA_3 positive regions and Ag-NORs in the karyotypes of *A. obscurus* and *A. fimbriatus* (Fig. 2a–f). Karyotypes of both species had presented CH blocks associated with Ag-NORs sites, reflecting GC-richness in these heterochromatics clusters. In addition, positive CMA_3 signals were observed in the pericentromerics regions of certain autosomes, particularly in pairs 1, 2 and 6 of *A. obscurus* and in pair 6 of *A. fimbriatus* (Fig. 2b,e). On the other hand, a uniform pattern was observed in all the chromosomes after DA/DAPI staining (Fig. 2c–d).

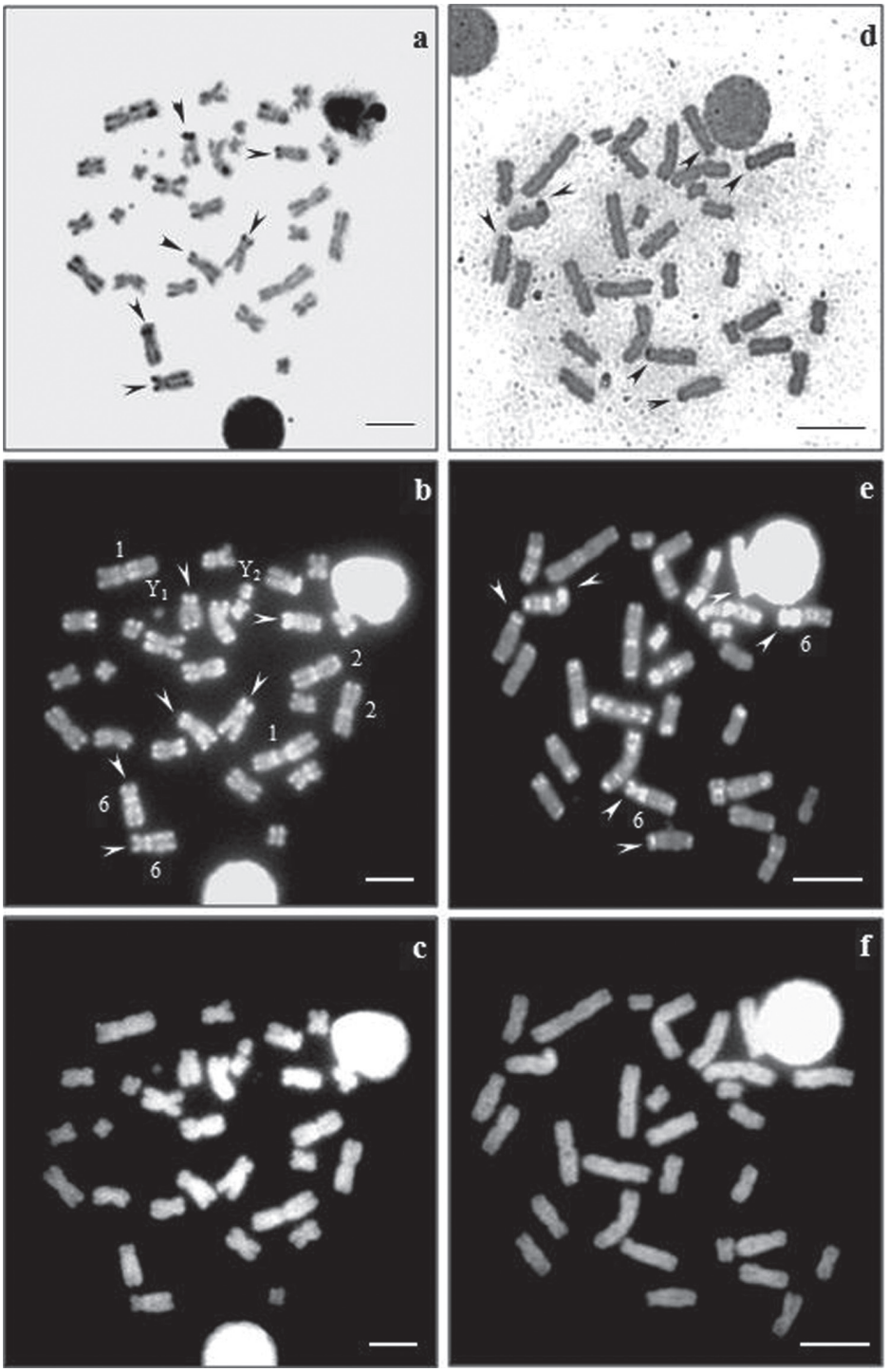

Figure 2. Sequential staining of *A. obscurus* **(a–c)** and *A. fimbriatus* **(d–f)** karyotypes with $AgNO_3$/CMA_3/DA/DAPI. **(a,d)** Ag-NORs, **(b,e)** CMA_3, **(c–f)** DA/DAPI. Bar = 5 μm.

Table 1. Heterochromatin pattern in chromosomal complement in *Artibeus* species

Species	C-banding				
	Pericentromeric	Terminal	Interstitial	Dispersed	Distal
A. obscurus	+	5p, 6p, 7p, 9p	1*, 2q, 5q, 6q	Y_1 e Y_2	-
A. fimbriatus	+	5p, 6p, 7p	-	Y_1 e Y_2	-
A. lituratus	+	5p, 6p, 7p	6q	Y_1 e Y_2	-
A. planirostris	+	5p, 6p, 7p, 9p	1q, 2q, 5q, 6q	Y_1	Y_2

(p) = short arm; (q) = long arm; * = both p and q; + = all chromosomes; - = absent

Table 2. Frequency analyzes of active NORs in the large species of genus *Artibeus*.

Species	Active NOR number per cell						Total of cells analyzed
	1	2	3	4	5	6	
A. obscurus	0	17	53	71	12	16	169
A. fimbriatus	0	14	40	58	27	22	161
A. lituratus	0	7	30	36	22	24	119
A. planirostris	0	14	28	47	11	25	125
Total	0	52	151	212	72	87	574
(%) total	0	9.06	26.31	36.94	12.54	15.16	

Discussion

Our data regarding diploid number, chromosome morphology and sex determination system obtained for *Artibeus obscurus*, *A. fimbriatus*, *A. lituratus* and *A. planirostris* karyotypes are in agreement with those previously described in the literature (Baker and Hsu 1970, Gardner 1977, Baker et al. 2003). The autosomal complements presented are morphologically similar to other, except by length of the sex chromosomes Y_1 and Y_2, which varies from punctiform elements to well-defined acrocentric chromosomes, as firstly described by Hsu et al. (1968) for *A. lituratus*, *A. jamaicensis* Leach, 1821 and *A. toltecus* Saussure, 1860.

The multiple sex chromosome system XY_1Y_2 has been widely reported within the genus *Artibeus*, e.g. *A. aztecus* Andersen, 1906, *A. glaucus* Thomas 1893, *A. toltecus*, *A. concolor, A. cinereus* Gervais, 1856*, A. hirsutus* Andersen, 1906*, A. inopinatus* Davis et Carter, 1964 and *A. jamaicensis*, and for other 23 species of family Phyllostomidae, as predominant type of sex-determining mechanism in this group (Baker and Hsu 1970, Varella-Garcia et al. 1989, Wetterer et al. 2000, Baker et al. 2003, Noronha et al. 2009). In mammals, this sexual system has been reported in marsupials, insectivores (shrews), carnivores (mongoose), rodents (hamsters) and in artiodactyls (gazelles) (reviewed in Gruetzner et al. 2006). Its origin involves a single sex chromosome-autosome translocation, which in meiosis leads to one sexual trivalent structure formed by XY_1Y_2 (Rodrigues et al. 2003, Noronha et al. 2004).

The CH distribution was evaluated and intercompared in the large *Artibeus* and with others phyllostomatids, pointing out an extensive similarity of CH pattern local-

ized in the pericentromeric region (Rodrigues et al. 2000, Barros et al. 2009, Sbragia et al. 2010). Additionally, CH blocks were also found in the terminal region of chromosome pairs 5, 6 and 7 that has been considered a characteristic shared by subfamily Stenodermatinae (Souza and Araújo 1990, Santos and Souza 1998b, Silva et al. 2005).

On the other hand, a particular set of CH blocks was observed in *A. obscurus*, *A. fimbriatus*, *A. lituratus* and *A. planirostris*. This finding allowed the individualization and differentiation of each species for karyotype comparison (Fig. 1). *A. fimbriatus* and *A. lituratus* karyotypes showed a closer CH distribution differing only by one heterochromatin block. Furthermore *A. obscurus* and *A. planirostris* karyotypes presented more interstitial heterochromatin.

The occurrence of intrageneric variation on CH distribution had been described only in sporadic cases among phyllostomatids whose extensive karyotypic conservation is widely known. In turn, the genus *Artibeus* is widely cited as a chiropteran group that exhibits low rate of karyotype evolution whereas: (1) most of species had same diploid number (30/31) and (2) G-banding patterns are essentially identical (Baker and Bickham 1980, Baker et al. 2003).

The other parameter evaluated intercomparison was the NORs localization by silver staining. The Ag-NORs were situated on the subtelocentric autosomes 5, 6 and 7 of all species. The data obtained for *A. lituratus*, *A. planirostris* and *A. fimbriatus*, together with the new data of *A. obscurus*, were similar those described by Santos et al. (2002). These authors employed FISH with 18S ribosomal probe allied to silver staining to investigate the precise localization of rDNA sites, and discovered a non-correlation between the number and distribution of the NORs in *A. cinereus* Gervais, 1856, being the first report on silent NORs in bats. They also had distinguished two rDNA sites patterns for *Artibeus* genus: 1) in the distal regions of the short arms of pairs 5, 6 and 7 (*A. lituratus*, *A. jamaicensis* Leach, 1821 and *A. fimbriatus*) and 2) in the interstitial region of the long arms of pairs 9, 10 and 13 (*A. cinereus*). In addition, *A. fimbriatus* had one NOR in the interstitial region on the long arm of pair 5, that it was not observed in this work, which may indicate a chromosomal polymorphism for this species.

As only active NORs could be visualized in our data, the variation in Ag-NORs activity for cell was also investigated (Table 2). In the most of cells analyzed (> 500), the frequency of active NORs was 3 or 4 black spots (26.31 to 36.94 %). Such variability is in accordance with other studies on a NOR sites activity in Phyllostomidae bats that presents multiple NORs (Morielle and Varella-Garcia 1988, Souza and Araújo 1990, Santos et al. 2002).

The association between NORs and CH by GC-specific fluorochromes staining presented in this work for *A. obscurus* and *A. fimbriatus*, has also been reported to *A. lituratus*, *A. jamaicencis*, *Desmodus rotundus* Geoffroy, 1810, *Diphylla ecaudata* Spix, 1823 and *Lonchorhina aurita* Tomes, 1863. On the other hand, *Carollia perspicillata* Linnaeus, 1758, *Molossus molossus* Pallas, 1766*, M. ater* Peters, 1865*, Molossops planirostris* Peters, 1865, *Phyllostomus discolor* Wagner, 1843 and *Trachops cirrhosus* Spix, 1823 NORs and CH were CMA_3 neutral. The reason for that is probably in heterogeneity of

base composition of the intergenic regions related to NORs. In some cases, the triple staining with CMA_3/DA/DAPI has also enhanced the patterns of R-bands with $CMA_{3,}$ an uniform staining with DA/DAPI or a weak G-banding pattern, as it has been observed in some bat's families (Santos and Souza 1998a, 1998b, Santos et al. 2001, Leite-Silva et al. 2003, Barros et al. 2009).

Conclusion

Classical and molecular cytogenetic markers, associated to taxonomic studies, have provided a better understanding of phylogenetic relationships and the mechanisms responsible for chromosomal divergence in the different taxa in the order Chiroptera. Cytogenetic analysis of all Brazilian species of the subgenus *Artibeus* allowed us to reveal the conservative and specific chromosomal features among their karyotypes. Furthermore, it was possible to identify intrageneric and interespecific divergences in a group that up to today has been characterized by showing extensive karyotypic conservation. The cytogenetic techniques herein employed, demonstrated the usefulness of C-banding in the identification and correct individualization of the large *Artibeus* that live sympatrically in the northeastern of Brazil, thus providing an important tool in the cytotaxonomic differentiation of this genus.

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Appendix

Família Phyllostomidae

Artibeus obscurus
M 318 (3214) – Saltinho (Rio formoso)
M 319 (3206) – Saltinho (Rio formoso)
M 336 (3235) – Saltinho (Rio formoso)
M 337 (3238) – Saltinho (Rio formoso)
M 340 (3216) – Saltinho (Rio formoso)
M 344 (3237) – Saltinho (Rio formoso)
M 359 (3230) – Saltinho (Rio formoso)
M 381 (3181) – Saltinho (Rio formoso)
M 397 (3186) – Dois irmãos (Recife)
M 455 (3189) – Saltinho (Rio formoso)
M 478 (3179) – Saltinho (Rio formoso)

A. fimbriatus
M 346 (3215) – Saltinho (Rio formoso)
M 382 (3184) – Saltinho (Rio formoso)
M 395 (3177) – Dois irmãos (Recife)
M 453 (3175) – Saltinho (Rio formoso)
M 479 (3192) – Saltinho (Rio formoso)

A. lituratus
M 221 (3418) – Igarassu
M 379 (3178) – Saltinho (Rio formoso)
M 446 (3191) – Saltinho (Rio formoso)
M 454 (3182) – Saltinho (Rio formoso)
M 475 (3188) – Saltinho (Rio formoso)
M 476 (3180) – Saltinho (Rio formoso)

A. planirotris
M 118 (3424) – Igarassu
M 124 (3212) – Igarassu
M 137 (3423) – Aldeia (Camaragibe)
M 188 (3428) – Igarassu
M 262 (3220) – Água Preta (Fazenda Camarão)
M 263 (3218) – Água Preta (Fazenda Camarão)
M 393 (3202) – Dois irmãos (Recife)
M 400 (3176) – Dois irmãos (Recife)
M 401 (3196) – Dois irmãos (Recife)

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RESEARCH ARTICLE

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Three sympatric karyomorphs in the fish *Astyanax fasciatus* (Teleostei, Characidae) do not seem to hybridize in natural populations

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Abstract

Ninety individuals of the characid fish *Astyanax fasciatus* (Cuvier, 1819) were collected at Água da Madalena stream (Botucatu, São Paulo, Brazil) and analyzed for diploid chromosome number 2n and karyotype composition as well as for the chromosomal location of the 5S and 18S ribosomal DNA (rDNA). Whereas no chromosome differences were associated with sex, three different karyomorphs with diploid chromosome numbers 2n=46, 2n=48 and 2n=50 were found. No intermediate 2n numbers were discovered. The 2n=50 karyomorph showed some differences in 18S rDNA location compared to the two other karyomorphs. Finally, all specimens with the 2n=46 karyomorph showed the presence of a partly heterochromatic macro supernumerary chromosome, which was absent in all individuals with the two other karyomorphs. All these results suggest that indviduals of the three different karyomorphs are not likely to hybridize in the examined populations. Our findings strongly suggest the presence of three separate species (*sensu* biological species concept) easily diagnosed on the basis of differences in the diploid chromosome numbers and other chromosomal markers.

Keywords
fish cytogenetics, chromosome banding, rDNA, sympatric differentiation, B chromosome

Introduction

The genus *Astyanax* (Baird et Girard, 1854) is one of the most diversified among Neotropical characid fishes. Indeed, this genus shows an extensive morphological diversification and a highly complex taxonomy. Moreover, a number of species actually appear to be "complexes of species" with low morphological differentiation but high variation at other levels, e.g. chromosome number and morphology (Morelli et al. 1983). The first *Astyanax* "species complex" was suggested by Moreira-Filho and Bertollo in *Astyanax scabripinnis* Jenyns, 1842 (Moreira-Filho and Bertollo 1991). Other cases were reported in the Neotropical fish *Corydoras aeneus* Gill, 1858 (Turner et al. 1992), *Hoplias malabaricus,* Bloch 1794 (Bertollo et al. 1997), and *Gymnotus carapo* Linnaeus, 1758 (Milhomem et al. 2008).

Astyanax fasciatus is another species that seems to form a "species complex" since the available information points towards the existence of several karyomorphs with the diploid chromosome numbers 2n=45, 2n=46, 2n=47, 2n=48 and 2n=50 (Table 1). The karyotype most frequently cited in the literature is 2n=48 (Table 1), but the known geographical range is actually larger for the karyomorphs 2n=50 and 2n=46 (Figure 1).

B chromosomes are supernumerary elements previously reported in the karyotypes of several *Astyanax* species (Moreira-Filho et al. 2004). In *A. fasciatus*, B chromosomes have been reported only for the 2n=46 karyomorph in a population at the Sao Francisco River basin (Moreira-Filho et al. 2001).

Table 1. Diploid numbers (2n) recorded in populations of *Astyanax fasciatus* complex.

Species as originally reported	River/basin	2n	B chrom.	Reference
A. fasciatus	Mogi Guaçu river/Paraná	45		14
A. fasciatus	Mogi Guaçu river/Paraná	46		2,4,9,10,11,18
A. fasciatus	Mogi Guaçu river/Paraná	47		14,11
A. fasciatus	Mogi Guaçu river/Paraná	48		11,18
A. fasciatus	Piracicaba river/Paraná	48		7
A. fasciatus	Piracicaba river/Paraná	46		23
A. fasciatus	Tietê river/Paraná	46		5
A. fasciatus	Riacho Águas da Madalena/Paraná	50		23
A. fasciatus	Águas da Madalena stream/Paraná	48		23

Species as originally reported	River/basin	2n	B chrom.	Reference
A. fasciatus	Águas da Madalena stream/Paraná	46	+	23
A. fasciatus	Paranapanema river/Paraná	46		6
A. fasciatus	Paranapanema river/Paraná	50		13
A. fasciatus	Paraíba river/Paraná	48		3,5,9
A. fasciatus	Paiol Grande river/Paraná	48		9,17
A. fasciatus	Barra funda river/Paraná	46		9
A. fasciatus	Passa Cinco river/Paraná	46		9,11
A. fasciatus	Sapucaí river/Paraná	48		12
A. fasciatus	Araguari river/Paraná	46		16
A. cf. fasciatus	Juquiá river/Paraná	48		2
A. fasciatus	Meia Ponte river/Araguaia	46		1
A. prope fasciatus	Córrego Fundo stream/Araguaia	50		8
A. fasciatus	Araras river/Paraná	48		22
A. fasciatus	Patos river/Paraná	48		22
A. fasciatus	Três Bueiros river/Paraná	48		22
A. fasciatus	Almas river/Paraná	48		22
A. prope fasciatus	Tibagi river/Paraná	48		19
A. prope fasciatus	Tibagi river/Paraná	49		19
A. prope fasciatus	Tibagi river/Paraná	50		19
A. fasciatus	São Francisco river/São Francisco	48		7,9,17,22
A. fasciatus	São Francisco river/São Francisco	46	+	7
A. fasciatus	Três Marias/São Franscisco	46	+	15
A. fasciatus	Contas river/Leste	48		21
A. fasciatus	Mineiro do Costa stream/Leste	48		21
A. fasciatus	Preto do Costa river/Leste	48		21

References: 1. Jim and Toledo (1975); 2. Morelli et al. (1983); 3. Moreira-Filho and Bertollo (1986); 4. Paganelli (1990); 5. Justi et al. (1990); 6. Reganham and Giuliano-Caetano (1990); 7. Justi (1993); 8. Centofante and Vênere (1995); 9. Heras and Moreira-Filho (1996); 10. Daniel-Silva (1996); 11. Heras and Moreira-Filho (1997); 12. Swerts et al. (1998); 13. Vale and Martins-Santos (1999); 14. Daniel-Silva and Almeida-Toledo (2001); 15. Moreira-Filho et al. (2001); 16. Torres-Mariano and Morelli (2006); 17. Abel et al. (2006); 18. Pazza et al. (2006); 19. Artoni et al. (2006); 21. Medrado et al. (2008); 22. Peres et al. (2009); 23. Ferreira-Neto et al. (present study).

In this study we analyzed the 2n=46, 2n=48 and 2n=50 *A. fasciatus* karyomorphs by examining a high number of individuals living in sympatry. The absence of intermediate chromosome numbers, the restriction of B chromosomes to the 2n=46 karyomorph and the absence of heteromorphism for the 18S rDNA sites (which differ among some karyomorphs) strongly suggest that the abovementioned karyomorphs are not likely to hybridize and are thus three separate species (*sensu* biological species concept).

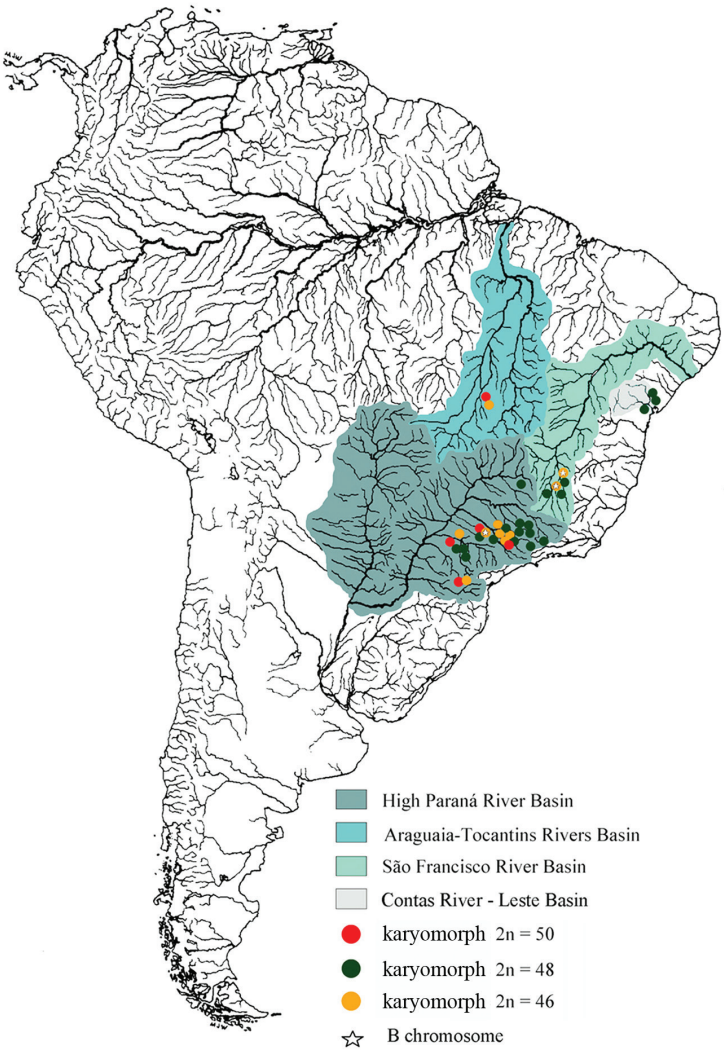

Figure 1. Map of South America highlighting the hydrographic basin of the Paraná, Araguaia-Tocantins, Contas and São Francisco Rivers. The natural distribution of *Astyanax fasciatus* and the presence of B chromosomes are represented according to the legends.

Material and methods

Ninety *A. fasciatus* specimens (56 females and 34 males) were collected at a 3.000 m^2 area of the Água da Madalena stream, Botucatu, SP, Brazil (22°59'23"S 48°25'31"W). The specimens were anaesthetized in benzocaine (1%) and, after collecting tissue samples for chromosome analysis, fixed in 10% formol and preserved in 70% alcohol. After identification, the specimens were deposited in the Museum of Biology and Laboratory of Fish Genetics, UNESP, Botucatu, São Paulo, Brazil. Chromo-

some preparations were obtained from anterior kidney cells and used for the conventional air drying technique (Foresti et al. 1981). The chromosomal location of active nucleolus organizer regions (NORs) was detected using the silver nitrate staining technique (Howell and Black 1980). Mapping of the ribosomal DNA (rDNA) was performed by fluorescent *in situ* hybridization (FISH) according to Pinkel et al. (1986). The 18S and 5S rDNA probes were obtained from the fish *Prochilodus argenteus* Spix and Agassiz, 1829 (Hatanaka and Galetti Jr. 2004) and *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti Jr. 1999), respectively. The 5SS probe was labeled with biotin 14-dATP by nick translation following manufacturer's instructions (Bionick Labelling System - Invitrogen). Hybridization was detected with avidin-FITC and the signals were amplified with biotinylated anti-avidin. The 18S probe was labeled with digoxigenin 11-dUTP (Roche Applied Sciences) by PCR (Polymerase Chain Reaction) and hybridization signals were detected using anti-digoxigenin-rhodamine.

Metaphase chromosomes were counterstained with DAPI and analyzed under optical light microscope (Olympus BX61). Images were captured using the Image-Pro Plus 6.0 software (Media Cybernetics). To build karyograms, chromosome morphology was determined according to the arm ratio limits established by Levan et al. (1964), and chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a), and were arranged in order of decreasing size.

Results

The cytogenetic analysis of the 90 specimens of *Astyanax fasciatus* revealed the presence of three different karyomorphs in the sample, showing 2n=46, 2n=48 and 2n=50 chromosomes (Table 2 and Fig. 2 a, c and e, respectively), and with fundamental numbers (NF= number of chromosome arms) equal to 84, 86 and 90, respectively. The anatomical sex of the fish, determined by visual examination of the gonads, was not associated with the karyotypic differences, so that males and females were found with all the three cytotypes described here. Interestingly, all the specimens with the 2n=46 karyomorph showed the presence of one mitotically stable macro B chromosome (Fig. 2 a, b), whereas the specimens of the 2n=48 and 2n=50 karyomorphs lacked it. No individual with intermediate odd chromosome numbers was found.

Table 2. Cytogenetic studies in *Astyanax fasciatus* karyotype composition and location of the 5S and 18S rDNA in the chromosomes of the individuals analyzed.

Cytotypes 2n / NF	Number of specimens	Chromosome	rDNA 18S/5S	Number of B Chromosomes		NOR
	Female Male	Formulae	location (pairs)	females	males	(pairs)
2n = 46, NF=84	12 10	5m+8sm+6st+4a	8,15,16,20,21, B/3,20	1	1	9,10
2n = 48, NF=86	10 9	5m+8sm+6st+5a	8,12,19,20,21/3,20	0	0	9,10
2n = 50, NF=84	41 32	5m+8sm+4st+8a	5,8,12,18,22/3,21	0	0	9,10

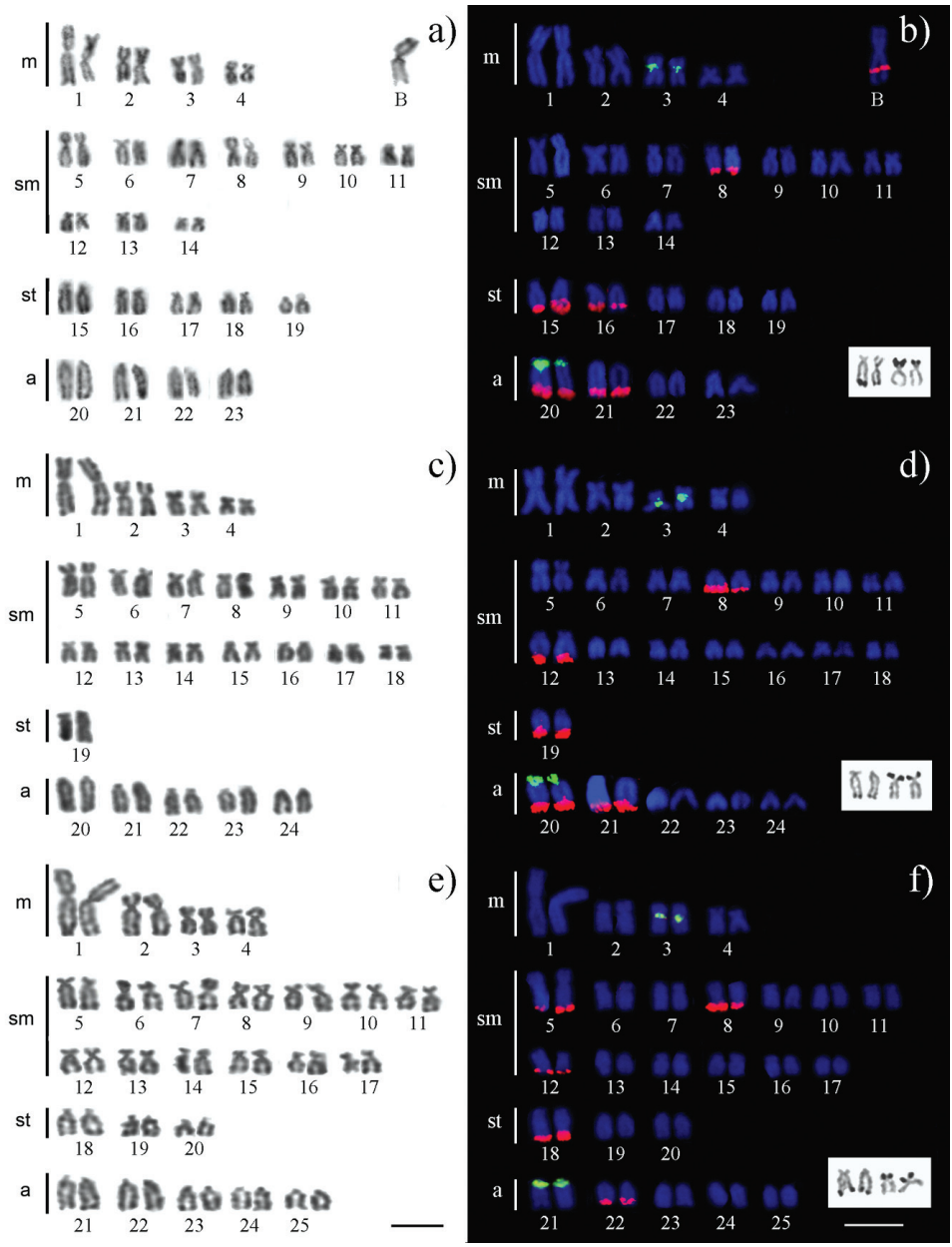

Figure 2. Karyotypes of *Astyanax fasciatus* (2n=46, 48 and 50 chromosomes) deduced after conventional Giemsa staining (a, c, e) and double FISH using 5S (green) and 18S rDNA (red) probes. The chromosomes bearing the Ag-NORs are boxed in b, d and f. Bar = 10 μm.

Physical mapping of the rDNA showed remarkable conservation of the 5S rDNA sites, which were present in two chromosome pairs, i.e. Nos. 3 and 20 (21 in the 2n=50 karyomorph) (Fig. 2 b, d, f).

The silver staining technique revealed the presence, in all cells, of Ag-NORs on two sm chromosome pairs of the three karyomorphs. One Ag-NOR was located at the distal region of the p arm and the other at the q arm (Figs 2 b, d, f, details). FISH analysis, however, showed the presence of 18S rDNA at the distal regions of five chromosome pairs, but no FISH signal was observed on the short arm of any chromosome (Fig. 2 b, d, f). This points towards the presence of a small number of 18S rRNA genes in the short arm of the sm chromosome.

Double FISH showed the presence of a chromosome pair, No. 20 in the 2n=46 and 2n=48 karyomorph, carrying both 5S (proximal) and 18S (distal) rDNA, but no chromosome carried both kinds of rDNA in the 2n=50 karyomorph (Fig. 1f). All six specimens analyzed by FISH were homomorphic for the chromosome No. 20 which carries both rDNA types. Remarkably, FISH mapping showed the presence of an interstitial cluster of 18S rDNA in the long arm of the B chromosome found in the 2n=46 karyomorph. Nevertheless, this rDNA cluster was never detected by silver staining.

Discussion

Sympatry and syntopy for several cytotypes have been reported in *Astyanax* species, such as *A. scabripinnis* (Souza and Moreira-Filho 1995) and *A. fasciatus* (Pazza et al. 2006, 2008). A review of all the published data on *A. fasciatus* (summarized in Table 1 and Figure 1) indicates that the three karyomorphs reported in this work show broad geographical distribution, and sympatry can be observed for at least two different karyomorphs in four river basins, i.e. High Paraná, Araguaia-Tocantins, Contas and São Francisco. In addition, B chromosomes have been previously reported in two other populations from the São Francisco River basin although, in that case, the number of B chromosomes showed variation among individuals (Justi 1993, Heras and Moreira-Filho 1997, Moreira-Filho et al. 2001). One evidence in support of hybridization events between karyomorphs was reported by Artoni et al. (2006), who found an individual with 49 chromosomes which might be a hybrid between the 48 and the 50 cytotypes in the Tibagi River (High Paraná River Basin, Ponta Grossa, Paraná, Brazil). The other evidence supporting hybridization between karyomorphs was reported by Pazza et al. (2006, 2008) who identified karyomorphs with 2n=45, 46, 47 and 48 chromosomes in individuals caught along the Mogi-Guaçu river (High Paraná River Basin, Cachoeira de Emas, São Paulo, Brazil). In this last case, the 2n=47 chromosomes karyomorph could have resulted from hybridization between the 46 and 48 cytotypes, whereas the presence of three individuals with 45 chromosomes could suggest the possible existence of a karyomorph with 2n=44 in that river.

Our present analysis of a large sample of individuals caught in the same stream shows syntopic occurrence of three *A. fasciatus* karyomorphs with discrete chromosome numbers 2n=46, 2n=48 and 2n=50. The absence of intermediate chromosome numbers, the presence of B chromosomes in only one of these karyomorphs (2n=46),

and the absence of apparent heteromorphism for the chromosome 20, suggests that the three karyomorphs do not hybridize in this stream. In such case, the data point out towards the possibility that these three karyomorphs actually correspond to three cryptic species, thus supporting the hypothesis that *A. fasciatus* is in fact a species assemblage, i.e. several species were included under the nominal name of *A. fasciatus* (Artoni et al. 2006).

The B chromosome found in *A. fasciatus* is large and metacentric. Interestingly, both characteristics seem common to all the B chromosomes described *Astyanax* species including *A. scabripinnis* (Salvador and Moreira-Filho 1992, Maistro et al. 1992, Souza and Moreira-Filho 1995, Vicente et al. 1996, Mizoguchi and Martins-Santos 1997, Vicari et al. 2010, 2011), *A. eigenmanniorum* Cope, 1894 (Fauaz et al. 1994), *A. schubarti* Britski, 1964 (Moreira-Filho 2001), *A. fasciatus,* (Moreira-Filho 2001) and *A. bockmanni,* Vari & Castro 2007 (Daniel personal communication). Such morphological similarity would support the hypothesis of a common origin of the *Astyanax* B chromosomes (Moreira-Filho et al. 2001). Nonetheless we show here, for the first time, the presence of rDNA interstitially located in the p arm of the B chromosome in *A. fasciatus* individuals. The apparent absence of rDNA in the q arm may seem incompatible with the isochromosome origin previously suggested for some of these Bs, e.g. in *A. scabripinnis* (Mestriner et al. 2000). However, the presence of a small, bellow FISH sensitivity, amount of rRNA genes is also possible. A genus-wise investigation of the presence and distribution of the rDNA in *Astyanax* species may be very useful for inferring the origin of the B chromosome. To this end, a comparison of rDNA sequences among the A and B chromosomes of several species would be very informative.

Since silver staining of metaphasic chromosomes reveals only those NORs that were active in the previous interphase (Hsu et al. 1975), it seems that the 18S rDNA contained in the B chromosome is usually inactive. Inactivity of the rDNA seems thus a widespread and general feature of the B chromosomes as it was previously reported in several phylogenetically distant species including the grasshopper *Eyprepocnemis plorans* Charpentier, 1825 (Cabrero et al. 1997, Bakkali et al. 2001), the black rat *Rattus rattus* Linnaeus, 1758 (Stitou et al. 2000) and the fish *Haplochromis obliquidens* Hilgendorf, 1888 (Poletto et al. 2010). Nonetheless, active rRNA genes have ocasionally been reported in some B chromosomes of species like the grasshopper *E. plorans* (Teruel et al. 2007, 2009) and the rodents *Akodon montensis* Thomas, 1913 and *Oryzomys angouya* Fischer, 1814 (Silva et al. 2004).

The higher number of rDNA clusters (10) than silver stained NORs (4) suggests the inactivity of some rDNAs in most cells. It would therefore be interesting to ascertain whether this phenomenon is facultative or constitutive. Silver nitrate may also bind to other proteins present in the nuclei, implying that some chromosome structures visualized by silver nitrate may not correspond to ribosomal genes (Dobigny et al. 2002). The detection of a higher number of ribosomal genes using FISH against 18S or 28S sequences than using silver nitrate staining is a common result having been reported in *Astyanax scabripinnis* (Ferro et al. 2001), *Salmo truta* (Pendás et al. 1993),

Colossoma macropomun Cuvier, 1816, *Piaractus brachypomus* Cuvier, 1818 and their interspecific hybrids (Nirchio et al. 2003), *Hyphessobrycon anisitsi* Eigenmann, 1907 (Centofante et al. 2003), *Prochilodus lineatus* Valenciennes, 1836 (Jesus and Moreira-Filho 2003) and *Lebias fasciata* Valenciennes, 1821 (Tigano et al. 2004). Therefore, the chromosome pair that actually has the nucleolus organizer region is probably the first submetacentric pair (the one that shows both FISH and silver nitrate staining signals).

The presence of one B chromosome in all 22 individuals from the 2n=46 karyomorph is intriguing, since it departs from the usual interindividual variation which characterizes B chromosomes (Camacho 2005). On the other hand, Moreira-Filho et al. (2001) reported the presence of B chromosomes showing interindividual variation in number in 10% of the sample population analyzed at the Sao Francisco River basin. This suggests that the B chromosomes in *A. fasciatus* from the Água da Madalena stream (this report) might be in a state of stabilization. With complete elimination through one sex and complete drive through the other, the population dynamics of *Astyanax* B chromosome resembles the case of germ-line restricted chromosomes in the zebra finch (Itoh et al. 2009). The mechanism behind B chromosome stabilization in populations of the Água da Madalena stream appears therefore to be rather complex and its elucidation requires further population dynamics and chromosome transmission studies.

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RESEARCH ARTICLE

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Cytogenetic analysis in the *incertae sedis* species *Astyanax altiparanae* Garutti and Britzki, 2000 and *Hyphessobrycon eques* Steindachner, 1882 (Characiformes, Characidae) from the upper Paraná river basin

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Abstract

Cytogenetic analyses were accomplished in two populations of *Astyanax altiparanae* Garutti & Britzki, 2000 and one population of *Hyphessobrycon eques* Steindachner, 1882, considered *incertae sedis* in Characidae family. Two populations of *A. altiparanae* (Mogi-Guaçu and Tietê rivers) presented 2n=50, with the same karyotype formula: 6M+12SM+20ST+12A (FN=88). *H. eques* from Capivara river presented 2n=52 and karyotype formula 14M+16SM+4ST+18A (FN=86). In each karyotype, the nucleolus organizer regions were detected at the end of the short arm of a single medium-sized subtelocentric chromosome. The Chromomycin A_3 (CMA_3) marking is coincident for the NORs in chromosomes of the two species and present additionally in two different chromosomes of *A. altiparanae* thus showing interpopulation differences in this species. In *H. eques*, weak heterochromatic blocks in the position of centromeres and telomeres of most chromosomes and negative C-banding for the NOR bearing chromosome were visualized. The obtained results contribute both to the understanding of karyotype evolution of these species and to the clarifying their phylogenetic relationships.

Keywords

Ag-NOR, Chromomycin A_3, chromosomes, evolution, Neotropical fish

Introduction

The neotropical freshwater ichthyofauna is quite rich, including 71 families and more than 4,500 species known to be valid, according to the latest surveys (Reis et al. 2003, Nelson 2006, Buckup et al. 2007). The Characiformes are exclusively freshwater fish, distributed in America and Africa with highest diversity in the major Neotropical basins (Buckup 1998). Currently, this order comprises 1,674 valid species in 270 genera (Nelson 2006), a number probably underestimated (Vari 1998).

Astyanax Baird & Girard, 1854 and *Hyphessobrycon* Eigenmann, 1908 are genera from family Characidae with wide distribution throughout Central and South America, and previously placed in subfamily Tetragonopterinae (Géry 1977). Recently these groups were considered as *incertae sedis* in family Characidae together with about 100 genera (Lima et al. 2003, Nelson 2006). Regarding the phylogenetic relationship between the genera considered *incertae sedis*, Mirande (2009) proposed a hypothesis that suggests a close relationship between *Astyanax* and *Hyphessobrycon*, forming, along with other genera, the clade *Astyanax*.

Astyanax includes about 100 species, commonly known as lambari and piaba (Lima et al. 2003, Nelson 2006). Reviewing some species of *Astyanax*, Garutti and Britzki (2000) described *A. altiparanae*, a new species formally presented as *A. bimaculatus* Linnaeus, 1758, for the upper Paraná river basin. *A. altiparanae* presents a black humeral spot horizontally oval, two brown vertical bars located in the humeral region, and a black diamond spot at caudal peduncle that extends to the tip of median caudal rays (Lima et al. 2003).

The genus *Hyphessobrycon* with approximately 90 species is characterized by an interrupted lateral line, reaching up to 60 mm in total length, and some species present a remarkable color that may interest the aquarists (Lima et al. 2003, Nelson 2006). *H. eques* are commonly known as matogrossinhos, with distribution in the South American river basins, also in *La Plata* basin (Paraná-Paraguai-Uruguai-Prata) and in rivers of Amazonas basin (Lima et al. 2003, Nelson 2006).

Cytogenetic analysis of representatives of the genus *Astyanax* reveals that diploid numbers range from 2n=36 in *Astyanax schubarti* Britski, 1964 (Morelli et al. 1983) to 2n=50 chromosomes in *Astyanax paranae* Eigenmann, 1914 (Vicari et al. 2008). According to Galetti et al. (1994) the karyotype data from the genus *Astyanax* present a chromosomal variability between different species and characterize a karyotypic heterogeneity in evolution of this group due to structural chromosomal rearrangements, mainly of Robertsonian type. Additional levels of chromosomal evolution may be uncovered in intraspecific studies and with the use of various chromosome techniques. About twenty populations of *A. altiparanae* studied to date (Table 1) present 2n=50 chromosomes with differences in their karyotypic formula and in the number and position of nucleolar organizer regions (NOR) in the chromosomes (Fernandes and Martins-Santos 2004, Neto et al. 2009) (Table 1).

Little is known about the cytogenetic patterns for *Hyphessobrycon*, where only a few species have been karyotyped (Table 1), and for many of them, only the haploid num-

Table 1. Summary of cytogenetic data from Brazilian populations of *Astyanax altiparanae* and *Hyphessobrycon* spp. 2n - diploid number; M - metacentric; SM - submetacentric; ST - subtelocentric; A - acrocentric; FN = fundamental number; n-NORs - number of chromosomes with silver stained nucleolar organizer regions. * - cited as *Astyanax bimaculatus* Linnaeus, 1758. Location's list follows a geographical order.

Species	Location	2n	Karyotype	FN	n-NORs	Reference
Astyanax altiparanae Garutti and Britzki, 2000	Iguaçu river, Curitiba, PR (Iguaçu river basin)	50	6M+30SM+8ST+6A	94	2	Domingues et al. (2007)
	Jordão river, Manguerinha, PR (Iguaçu river basin)	50	6M+28SM+8ST+8A	92	2-4	Neto et al. (2009)
	Índios river, Cianorte, PR (Ivaí river basin)	50	6M+30SM+4ST+10A	90	10	Fernandes and Martins-Santos (2004)
	Tatupeba river, Maringá, PR (Ivaí river basin)	50	6M+26SM+6ST+12A	88	3	Fernandes and Martins-Santos (2006)
	*Meia Ponte river, Goiânia, GO (Meia Ponte river basin)	50	26M+24A	76	-	Jin and Toledo (1975)
	Feijão stream, São Carlos, SP (Mogi-Guaçu river basin)	50	6M+30SM+8ST+6A	94	1-3	Neto et al. (2009)
	*Mogi-Guaçu river, Pirassununga, SP (Mogi-Guaçu river basin)	50	10M+24SM+4ST+12A	88	-	Morelli et al. (1983)
	Mogi-Guaçu river, Pirassununga, SP (Mogi-Guaçu river basin)	50	6M+12SM+20ST+12A	88	2	Present study
	Paraná river, Porto Rico, PR (Paraná river basin)	50	6M+26SM+6ST+12A	88	2	Fernandes and Martins-Santos (2004)
	Claro river, Tamarana, PR (Paranapanema river basin)	50	10M+26SM+4ST+10A	90	1-4	Pacheco et al. (2001)
	Claro river, Tamarana, PR (Paranapanema river basin)	50	10M+24SM+4ST+12A	88	1-4	Pacheco et al. (2001)
	Claro river, Tamarana, PR (Paranapanema river basin)	50	10M+22SM+4ST+14A	86	1-4	Pacheco et al. (2001)
	Paranapanema river, Salto Grande, SP (Paranapanema river basin)	50	10M+22SM+6ST+12A	88	-	Daniel-Silva and Almeida-Toledo (2001)
	Keçaba river, Maringá, PR (Pirapó river basin)	50	6M+26SM+6ST+12A	88	1	Fernandes and Martins-Santos (2006)

Species	Location	2n	Karyotype	FN	n-NORs	Reference
Astyanax altiparanae Garutti and Britzki, 2000	Maringá river, Maringá, PR (Pirapó river basin)	50	6M+26SM+6ST+12A	88	3	Fernandes and Martins-Santos (2006)
	*São Francisco river, MG (São Francisco river basin)	50	-	-	-	Carvalho et al. (2002b)
	Tibagi river, Ponta Grossa, PR (Tibagi river basin)	50	6M+28SM+8ST+8A	92	2-3	Domingues et al. (2007)
	*Jurumirim river, SP (Tietê river basin)	50	-	-	-	Carvalho et al. (1998)
	Pântano stream, São Carlos, SP (Tietê river basin)	50	6M+28SM+4ST+12A	88	1-2	Neto et al. (2009)
	Tietê river, Penápolis, SP (Tietê river basin)	50	6M+12SM+20ST+12A	88	2	Present study
Hyphessobrycon anisitsi Eigenmann, 1907	Piracuama river (Paraíba do Sul river basin)	50	6M+16SM+12ST+16A	84	4	Centofante et al. (2003)
H. anisitsi Eigenmann, 1907	Perdizes stream (Paraná river basin)	50	6M+16SM+12ST+16A	84	3	Centofante et al. (2003)
H. flammeus Myers, 1924	Paraná river (Paraná river basin)	52	18M,SM+32ST+2A	102	-	Arefjev (1990)
H. reticulatus Ellis, 1911	Juquiá river, São Lourenço da Serra, SP (Paraná river basin)	50	14M+20SM+16ST	100	2	Carvalho et al. (2002a)
H. scholzei Ahl, 1937	Perdizes stream (Paraná river basin)	50	8M+20SM+8ST+14A	86	-	Arefjev (1990)
H. griemi Hoedeman, 1957	Itimirim river, Iguape, SP, Iguape river basin (Ribeira river basin)	48	-	-	-	Carvalho et al. (2002b)
H. herbertaxelrodi Géry, 1961	Itimirim river, Iguape, SP, Iguape river basin (Ribeira river basin)	52	10M,SM+42ST,A	-	-	Arefjev (1990)
H. reticulatus Ellis, 1911	Itimirim river, Iguape, SP, Iguape river basin (Ribeira river basin)	50	-	-	-	Carvalho et al. (2002b)
H. eques Steindchner, 1882	Capivara river, Botucatu, SP (Tietê river basin)	52	14M+16SM+4ST+18A	86	2	Present study

ber is known (Sheel 1973). Nevertheless, the chromosome number is variable among the species, between 2n=48 for *H. griemi* Hoedeman, 1957 (Carvalho et al. 2002a) and 2n=52 for *H. herbertaxelrodi* Géry, 1961 (Arefjev 1990) (Table 1). Karyotypic data for *H. eques* are not available in literature.

In the present study, we compare the karyotypes of two populations of *A. altiparanae* and one of *Hyphessobrycon eques* aiming to contribute to the increase of knowledge

about the patterns of diversity and evolution of karyotype in this *incertae sedis* group of Characidae.

Materials and methods

Specimens from two populations of *Astyanax altiparanae* and one of *Hyphessobrycon eques* were collected in streams from the upper Paraná river basin (Fig. 1). The individuals were anesthetized with benzocaine (5%) and then sacrificed for subsequent cytogenetic analysis. The processed specimens were fixed in 10% formalin and stored in 70% alcohol for further taxonomic studies. The preserved specimens were placed in the collection of fish from Laboratório de Biologia e Genética de Peixes (LBP), Departamento de Morfologia do Instituto de Biociências da UNESP, campus de Botucatu. Their deposit numbers are indicated below.

The following specimens were karyotyped: six males and four females of *A. altiparanae* from the Mogi-Guaçu river, Pirassununga, SP., Brazil (Mogi-Guaçu river basin, site 1 in the map, GPS: 21°55'37.6"S, 47°22'04.4"W) with number 1142 (LBP); four males and two females of *A. altiparanae* from the Tietê river, Penápolis, SP., Brazil (Tietê river basin, site 2, GPS: 21°18'46.1"S, 50°08'26.4"W) with number 2690 (LBP); and three males and two females of *H. eques* from the Capivara river, Botucatu, SP., Brazil (Tietê river basin, site 3, GPS: 22°53'57.6"S, 48°23'11.4"W) with number 2337 (LBP) (Fig. 1).

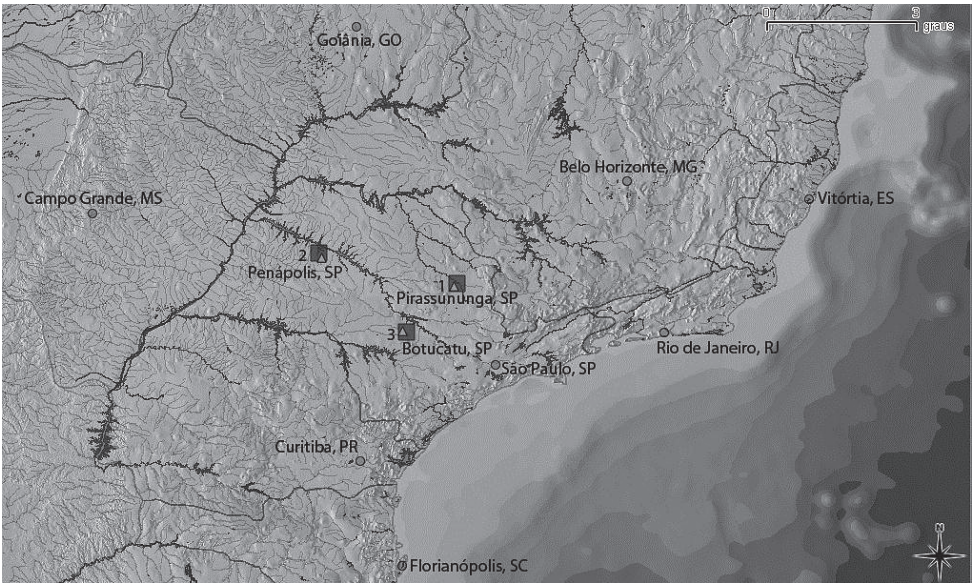

Figure 1. Map of the collection sites (squares) for the *Astyanax altiparanae* (**1, 2**) and *Hyphessobrycon eques* (**3**) in three rivers of the upper Paraná basin, São Paulo State (SP). Triangles refer to the neighboring cities and circles to the capitals of the states.

Metaphase chromosomes were studied on slide preparations made from kidney through the common air drying technique (Foresti et al. 1981), with the followed detection of the nucleolus organizer regions by the silver impregnation technique (Ag-NOR) from Howell and Black (1980), C-banding by the method of Sumner (1972), and flouorescent chromosome staining with Chromomycin A_3 (CMA_3) according to Schweiser (1976). The chromosome morphology was established based on the arm proportions about the centromere, as proposed by Levan et al. (1964), and the chromosome nomenclature commonly attributed to fish as metacentric (M), submetacentric (SM), subtelocentric (ST) and acrocentric (A) was used. Grouped correspondingly, the chromosomes were arranged in the hand constructed photo-karyograms of 3 fish populations studied (Figs 2, 3).

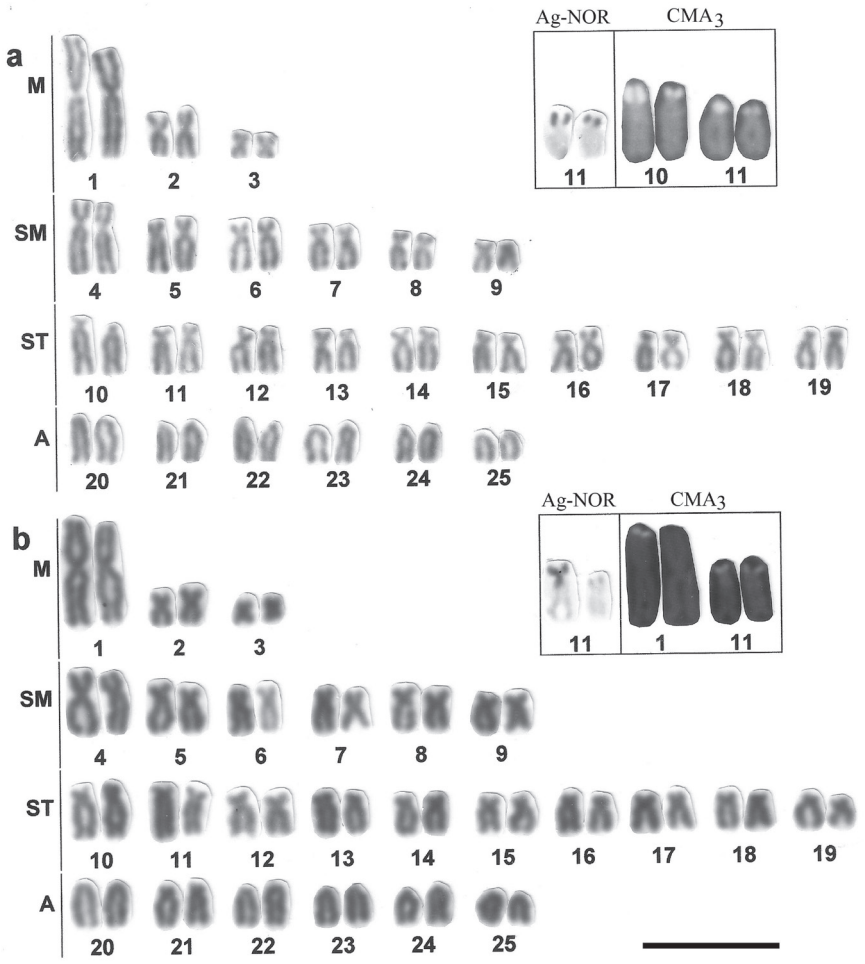

Figure 2. Karyograms showing chromosome morphology with the results of NOR-silver staining and Chromomycin A_3 (CMA_3) treatment (in a frame) on chromosomes of *Astyanax altiparanae* from Mogi-Guaçu river (**a**) and Tietê river (**b**). Bar = 5μm.

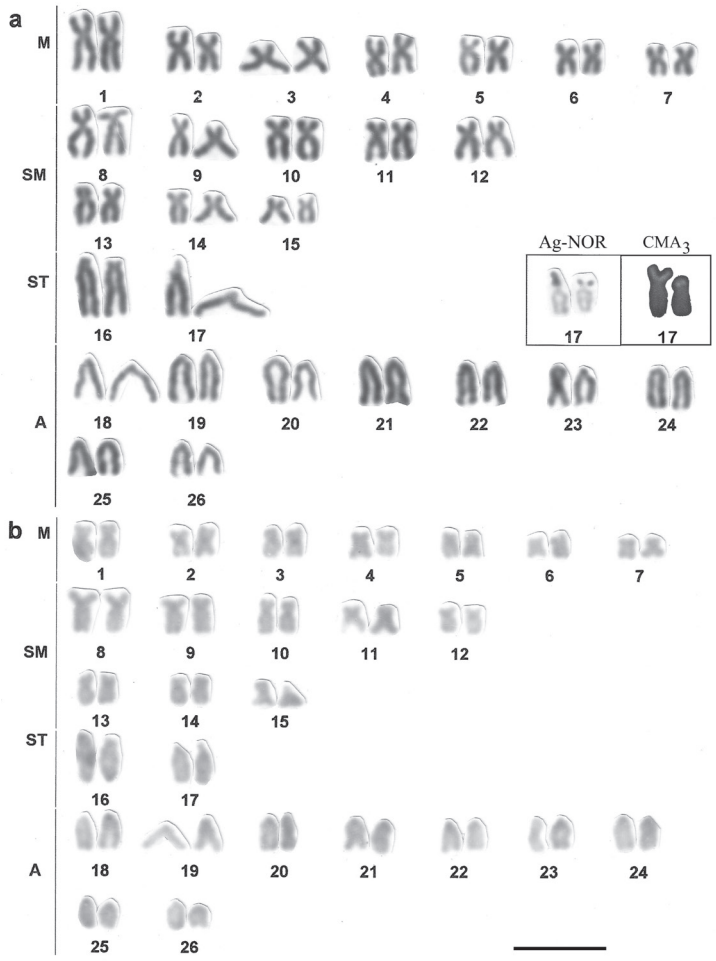

Figure 3. Karyotype of *Hyphessobrycon eques* showing (**a**) chromosome morphology with the results of NOR-silver and Chromomycin A_3 (CMA_3) staining in a frame, and (**b**) C-banding in fish individuals collected in the Capivara river. Bar = 5μm.

Results and discussion

Among the populations of *A. altiparanae* studied to date, all presented a diploid number of 50 chromosomes, and that is true for the two populations examined in the present study (Table 1). The karyogram of the species contains one big and two small metacentric pairs, a large group of submeta-subtelocentrics and not less than 6 acrocentric pairs (Figs 2a, b). The chromosome morphology did not show populational variations in samples from Mogi-Guaçu river and Tietê river in our data, which share the karyotype formula 6M+12SM+20ST+12A, and fundamental number is accordingly 88.

This chromosomal uniformity is, however, not common for populations from distinct hydrographic basins and even within the same basin, as other populations of

the rivers Tiete and Mogi-Guacu basins show considerable variation (Morelli et al. 19983, Neor et al. 2009) (see Table 1).

The intraspecific variety of chromosome formulae in this case is due to the variable content of each morphological group, from M to A, which interpretation may be difficult, however, without chromosome specific markers, still poorly available in ordinary fish cytogenetics. It should be stressed for this species that the presence of the big metacentric (pair M1) seems to be not only the common karyotype feature of species specific significance for *A. altiparanae*, but also a provisional phylogenetic marker. Taking into account the possibility of appearance of such a large bi-armed chromosome through Robertsonian fusion, it might focus to the karyotype relation between taxa under this study differing in 2n. Arm chromosome variation within and between the karyotypes might be caused by intrachromosomal changes such as pericentric inversion, centromeric shift, heterochromatin or NOR position.

The chromosome number is variable among the *Hyphessobrycon* species, ranging from 2n=52 chromosomes in *H. herbertaxelrodi* (Arefjev 1990) to 2n=48 in *H. griemi* (Carvalho et al. 2002b). Cytogenetic study of *H. eques* reveals a diploid number of 52 chromosomes and a karyotype formula with 14M+16SM+4ST+18A and FN=86. There are 9 acrocentric and 2 subtelocentric pairs in the karyogram and a large group of medium-sized submeta-metacentrics (Fig. 3). This is the first karyotype presentation of the species (Table 1).

Impregnation by silver nitrate reveals a single NOR location in a subtelocentric chromosome for populations of *A. altiparanae* and for the species *H. eques* (Figs 2, 3, Table 1). In *A. altiparanae*, the NOR marks are presented on a short arm of the subtelocentric pair 11 (Figs 2a, b, Table 1). In *H. eques*, the similar NOR bearing subtelocentric corresponds to the chromosome 17 in the species karyogram (Fig. 3).

The treatment with fluorochrome Chromomycin A_3 (CMA_3) was used to evidence NOR as regions associated with GC-rich DNA (Schmid and Guttenbach 1989). The Chromomycin A_3 treatment of chromosome preparations of *A. altiparanae* from Mogi-Guaçu river revealed marks in two chromosome pairs, one coincident with the NOR location in the pair 11, and the other on a short arm of the morphologically similar subtelocentrics of the pair 10 (Fig. 2a). However, in specimens from the Tietê river population, besides the coincident marking with the NOR bearing pair 11, a mark on one of the homologs of the largest pair (M1), was also detected (Fig. 2b).

NOR pattern variation has been reported for this species, from single to multiple NORs, which may characterize as intra- as inter-population variation in NOR location and chromosome morphology as well (Pacheco et al. 2001, Domingues et al. 2007, Neto et al. 2009). In our materials, the Chromomycin A_3 treatment data may suggest on possibility of the activity of extra number NORs above the single NOR pattern coincident with sylver staining in the upper Paraná populations studied.

In *Hyphessobrycon* species, too, there is a great variation of NORs appearing as single sites (Carvalho et al. 2002a) or multiple marked sites on chromosomes of the species (Centofante et al. 2003), that strengths the hypothesis of intensive chromosomal rearrangements in the group. C-banding identified in the genus, namely in *H. reticulatus*

Ellis, 1911 (Carvalho et al. 2002a) and *H. anisitsi* Eigenmann, 1907 (Centofante et al. 2003), appeared as small pericentromeric blocks in all chromosomes of the karyotype. In *H. anisitsi*, however, some chromosomes presented also terminal heterochromatic blocks, that was considered as indication on interpopulation differentiation. In view of uncertain chromosome identification, these data remain a preliminary information for further analyses only.

According to Mirande (2009), the *Astyanax* clade includes (along with all included species of *Astyanax*) *Markiana* Eigenmann, 1907, *Psellogrammus* Eigenmann, 1908, probably *Ctenobrycon* Eigenmann, 1908 and some *Bryconamericus* Eigenmann, 1907 and *Hyphessobrycon* taxa, suggesting that this highly diverse genus could be diagnosed as monophyletic with relatively few changes in its composition. Nevertheless the observed karyotype variations and poor supporting chromosome details, we could suggest rather close phylogenetic interrelation from the comparison of karyotypes of the two genera, *Astyanax* and *Hyphessobrycon*. It follows from an assumption of a Robertsonian change between the generic karyotypes viewed through changes of their morphology and 2n at maximal generic levels (50, 52) and proposedly common cytogenetic tools for multiple chromosomal differentiation of populations and species (NOR and C-banding patterns) though parallelisms cannot be excluded.

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RESEARCH ARTICLE

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Cytogeography of the *Humifusa* clade of *Opuntia* s.s. Mill. 1754 (Cactaceae, Opuntioideae, Opuntieae): correlations with pleistocene refugia and morphological traits in a polyploid complex

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Abstract

Ploidy has been well studied and used extensively in the genus *Opuntia* to determine species boundaries, detect evidence of hybridization, and infer evolutionary patterns. We carried out chromosome counts for all members of the *Humifusa* clade to ascertain whether geographic patterns are associated with differences in ploidy. We then related chromosomal data to observed morphological variability, polyploid formation, and consequently the evolutionary history of the clade. We counted chromosomes of 277 individuals from throughout the ranges of taxa included within the *Humifusa* clade, with emphasis placed on the widely distributed species, *O. humifusa* (Raf.) Raf., 1820 s.l. and *O. macrorhiza* Engelm., 1850 s.l. We also compiled previous counts made for species in the clade along with our new counts to plot geographic distributions of the polyploid and diploid taxa. A phylogeny using nuclear ribosomal ITS sequence data was reconstructed to determine whether ploidal variation is consistent with cladogenesis. We discovered that diploids of the *Humifusa* clade are restricted to the southeastern United States (U.S.), eastern Texas, and southeastern New Mexico. Polyploid members of the clade, however, are much more widely distributed, occurring as far north as the upper midwestern U.S. (e.g., Michigan, Minnesota, Wisconsin). Morphological differentiation, although sometimes cryptic, is commonly observed among diploid and polyploid cytotypes, and such morphological distinctions may be useful in diagnosing possible cryptic species. Certain polyploid populations of *O. humifusa* s.l. and *O. macrorhiza* s.l., however, exhibit introgressive morphological characters, complicating species delineations. Phylogenetically, the *Humifusa* clade forms two subclades that are distributed, respectively, in the southeastern U.S. (including all southeastern U.S. diploids, polyploid *O. abjecta* Small, 1923, and polyploid *O. pusilla* (Haw.) Haw., 1812) and the southwestern U.S. (including all southwestern U.S. diploids and polyploids). In addition,

tetraploid *O. humifusa* s.l., which occurs primarily in the eastern U.S., is resolved in the southwestern diploid clade instead of with the southeastern diploid clade that includes diploid *O. humifusa* s.l. Our results not only provide evidence for the polyphyletic nature of *O. humifusa* and *O. macrorhiza*, suggesting that each of these represents more than one species, but also demonstrate the high frequency of polyploidy in the *Humifusa* clade and the major role that genome duplication has played in the diversification of this lineage of *Opuntia* s.s. Our data also suggest that the southeastern and southwestern U.S. may represent glacial refugia for diploid members of this clade and that the clade as a whole should be considered a mature polyploid species complex. Widespread polyploids are likely derivatives of secondary contact among southeastern and southwestern diploid taxa as a result of the expansion and contraction of suitable habitat during the Pleistocene following glacial and interglacial events.

Keywords

Cactaceae, chromosome numbers, *Opuntia humifusa*, *Opuntia macrorhiza*, Pleistocene refugia, polyploid complex, polyploidy

Introduction

Ploidy has a long tradition of utility for illuminating species boundaries, hybrid zones, and interspecific relationships among plants (e.g., Stace 2000). Knowing the ploidal levels of taxa used in phylogenetic analyses can also aid in detecting potential hybridization events through incongruence in reconstructions using biparentally inherited nuclear loci (Ionta et al. 2007, Soltis et al. 2008). Researchers have frequently used cytological data to help understand species evolution and delimitations in the nopales or prickly pear cacti, i.e., the genus *Opuntia* (Pinkava and McLeod 1971, Pinkava et al. 1973, 1977, 1985, Weedin and Powell 1978, Pinkava and Parfitt 1982, Weedin et al. 1989, Pinkava et al. 1992, Powell and Weedin 2001, 2004). Subfamily Opuntioideae (*Opuntia* s.l., as previously recognized; Benson 1982) is known to have the highest number of polyploids in Cactaceae (Cota and Philbrick 1994, Pinkava 2002), and *Opuntia* s.s. is well known for interspecific hybridization (e.g., Grant and Grant 1982, Griffith 2003) and subsequent genome duplication (Pinkava 2002, L.C. Majure (LCM), R. Puente (RP), P. Griffith (PG), W.S. Judd (WSJ), P.S. Soltis (PSS), D.E. Soltis (DES) unpubl. data).

The significance of polyploidy in plant evolution and speciation has long been recognized (Stebbins 1940, 1950, 1971; Swanson 1957, DeWet 1971, Harlan and DeWet 1975, Grant 1981, Leitch and Bennett 1997, Ramsey and Schemske 1998, Adams and Wendel 2005, Tate et al. 2005, Doyle et al. 2008, Soltis and Soltis 2009, Jiao et al. 2011). As stated by Stebbins (1950), p. 369), "polyploidy … is one of the most rapid methods known of producing radically different, but nevertheless vigorous and well-adapted genotypes." Polyploidy also is considered one of the unequivocal means of true sympatric speciation (Futuyma 1998, Otto and Whitton 2000) and is considered to be common in plants (Stebbins 1940, DeWet 1971, Ramsey and Schemske 1998, Tate et al. 2005). For example, virtually all major clades of angiosperms have undergone one or more episodes of genome duplication (Soltis and Soltis 2009). Like-

wise, polyploidy is very important throughout Cactaceae (Pinkava 2002), and within *Opuntia* s.s., polyploids previously have been recorded in *Opuntia humifusa* (Raf.) Raf., 1820, and relatives (Bowden 1945a, b, Pinkava et al. 1985, Powell and Weedin 2004, Baker et al. 2009a, b, Majure and Ribbens in press) of the *Humifusa* clade (sensu LCM, RP, PG, WSJ, PSS, DES unpubl. data).

There are currently six species recognized in the *Humifusa* clade, *O. abjecta* Small, 1923, *O. humifusa*, *O. macrorhiza* Engelm., 1850, *O. pottsii* Salm-Dyck, 1849, *O. pusilla* (Haw.) Haw., 1812, and *O. tortispina* Engelm. & J.M. Bigelow, 1856 (Pinkava, 2003; LCM unpubl. data). The *Humifusa* clade is distributed widely from the western U.S. and northern Mexico (represented by *O. macrorhiza* s.l., *O. pottsii*, and *O. tortispina*) and throughout the eastern U.S. including the upper Midwest (e.g., Michigan, Minnesota, Wisconsin) and southern Ontario (Benson, 1982; represented by *O. abjecta*, *O. humifusa* s.l., *O. macrorhiza* s.l., and *O. pusilla*).

Opuntia humifusa s.l. is composed of numerous morphological entities that have been recognized in certain taxonomic treatments as different species (see Small 1933). Throughout its range, *O. humifusa* s.l. has been divided into as many as 14 taxa (Britton and Rose 1920, Small 1933, Benson 1982, Majure and Ervin 2008). Thus, *O. humifusa* s.l. is occasionally referred to as a species complex (Doyle 1990). Currently, two taxa are recognized in *O. humifusa* s.l. (*O. humifusa* var. *ammophila* (Small) L.D. Benson and *O. humifusa* var. *humifusa*; Pinkava 2003). Likewise, *Opuntia macrorhiza* has been divided into as many as 11 taxa (see Benson 1982). *Opuntia macrorhiza* was previously considered a variety of *O. humifusa* (see Benson 1962; see Table 1 for synonyms of *O. humifusa* s.l. and *O. macrorhiza* s.l. sampled in this study), *O. pottsii* was considered a variety of *O. macrorhiza*, and *O. tortispina* was placed in synonymy with *O. macrorhiza* (Benson 1982).

Opuntia pusilla has been divided into several species: *O. drummondii* Graham, 1841, *O. frustulenta* Gibbes, 1858, *O. impedita* Small, 1923, *O. pes-corvi* LeConte, 1857, and *O. tracyi* Britton, 1911 (Britton and Rose 1920, Small 1933); however, Benson (1982) placed them in synonymy under the name *O. pusilla*. *Opuntia triacantha* (Willd.) Sweet, 1826, also has been divided into several species, i.e., *O. abjecta* of the Florida Keys, *O. militaris* Britton & Rose, 1919, of Cuba, and *O. triacantha* from different parts of the Greater and Lesser Antilles (Britton and Rose 1920), but all of these have since been placed in synonymy within *O. triacantha* (Benson 1982). Phylogenetic and morphological studies have indicated that *O. abjecta* is not even in the same clade as *O. triacantha* (LCM, WSJ unpubl. data) and so here is treated as *O. abjecta*.

Contributing to the confusing taxonomic history of this clade is the high degree of morphological variation exhibited by most taxa, the lack of complete sampling throughout the range of the clade, the absence of cytological and phylogenetic evidence, reliance on poorly prepared and sparse herbarium collections (Majure and Ervin 2008, LCM unpubl. data), and hybridization and polyploidy (Benson 1982, Rebman and Pinkava 2001). Careful examination of morphological characters across the geographic range of the widely distributed *O. humifusa* s.l. and *O. macrorhiza*

Table 1. Synonyms of *O. humifusa* s.l. and *O. macrorhiza* s.l. sampled during this study.

***Opuntia humifusa* s.l.**	***Opuntia macrorhiza* s.l.**
Opuntia allairei	*Opuntia fusco-atra*
Opuntia ammophila	*Opuntia grandiflora*
Opuntia austrina	*Opuntia xanthoglochia*
Opuntia cespitosa	
Opuntia lata	
Opuntia nemoralis	
Opuntia pollardii	

s.l. reinforces the hypothesis that hybridization may have preceded the origin of geographical morphotypes, because morphological characters displayed by certain taxa appear to be introgessive between *O. humifusa* s.l. and *O. macrorhiza* s.l. (Table 2). For instance, *O. cespitosa* Raf., 1830, from the eastern U.S. and recently recognized by Majure and Ervin (2008), has yellow tepals that are basally tinged crimson- to orange-red, a characteristic typical of *O. macrorhiza* and occasionally *O. tortispina* from western North America (Benson 1982, Pinkava 2003, Powell and Weedin 2004), but the spine characters of *O. cespitosa* are typical of *O. humifusa* s.l. (see Majure and Ervin 2008).

Although chromosome counts have been reported for many of the *Opuntia* taxa from the southwestern U.S. and other areas (Stockwell 1935, Spencer 1955, Pinkava and McLeod 1971, Pinkava et al. 1973, 1977; Weedin and Powell 1978, Pinkava and Parfitt 1982, Pinkava et al. 1985, Weedin et al. 1989, Pinkava et al. 1992, Powell and Weedin 2001, Pinkava 2002, Negrón-Ortiz 2007, Segura et al. 2007, Baker et al. 2009a, b), few chromosome counts have been reported for taxa of *Opuntia* in the eastern and midwestern U.S. (Majure and Ribbens in press), and most of those taxa belong to the *Humifusa* clade. Bowden (1945a, b), Hanks and Fairbrothers (1969), Doyle (1990), and Baker et al. (2009 a, b) have all made counts of members of the *Humifusa* clade from the eastern U.S. Bowden (1945a, b), Doyle (1990), and Baker et al. (2009a) recorded diploid ($2n = 22$) and tetraploid ($2n = 44$) material of *O. humifusa* from the eastern U.S., and Bowden (1945a) recorded tetraploid ($2n = 44$) material of *O. impedita* (currently syn. of *O. pusilla*). Hanks and Fairbrothers (1969) recorded an aneuploid number for *O. humifusa* ($2n = 17, 19$) likely in error, since aneuploids are very rare in Cactaceae (Pinkava 2002). Majure and Ribbens (in press) recorded tetraploids of *O. humifusa* s.l. and *O. macrorhiza* s.l. from the Midwest, suggesting that the northernmost populations of those taxa are polyploid. *Opuntia macrorhiza, O. pottsii,* and *O. tortispina* have all been counted extensively in the southwestern U.S. (Pinkava and McLeod 1971, Pinkava et al. 1973, Pinkava et al. 1977, Pinkava et al. 1992, Pinkava et al. 1998, Powell and Weedin 2001, Powell and Weedin 2004), where *O. macrorhiza* and *O. pottsii* have been recorded exclusively as tetraploids, and *O. tortispina* has been recorded as either tetra- or hexaploid.

Chromosome counts reported for species in the *Humifusa* clade do not encompass all of the taxa within the range of the clade nor the wide distributions exhibited by sev-

Table 2. Selected taxa of *O. humifusa* s.l. and *O. macrorhiza* s.l. with morphological characters and corresponding ploidy. Polyploids often exhibit characters from more than one diploid taxon or characters of other polyploids, although certain characters (e.g., red glochids) have not been observed in any diploids analyzed thus far.

Taxon (ploidy)	Flower color	Cladode color	Spine barbedness/ Cladode disarticulation	Glochid color
***O. ammophila* (2*x*)**	Yellow	Dark green	Not barbed/no	Stramineous
***O. austrina* (2*x*)**	Yellow	Dark green	Barbed/yes	Stramineous
***O. cespitosa* (4*x*)**	Red-centered	Glaucous green	Not barbed/no	Red
***O. lata* (2*x*)**	Yellow	Dark green	Barbed/yes	Stramineous
***O. humifusa* (4*x*)**	Yellow	Dark green	Not barbed/no	Stramineous
***O. macrorhiza* (4*x*)**	Red-centered	Glaucous green	Not barbed/no	Red/yellow
***O. nemoralis* (4*x*)**	Yellow	Glaucous green	Barbed/yes	Yellow
***O. pollardii* (4*x*)**	Yellow	Dark green	Barbed/yes	Stramineous
O. xanthoglochia (2x)	Red-Centered	Glaucous green	Not barbed/no	Yellow

eral of the more common species. To further our understanding of species complexes and the evolution of polyploids within those complexes, cytological data are needed from the entire distribution of a given species (Babcock and Stebbins 1938, Stebbins 1942, Stebbins 1950). Thus, an in-depth study of the distribution of cytotypes and correlations between cytotypes and morphology is desperately needed in order to aid in the delimitation of potentially unrecognized and cryptic species and to elucidate relationships in the *Humifusa* clade.

Here we present chromosome counts for all taxa considered to be part of the *O. humifusa* complex and all taxa of the *Humifusa* clade (LCM, WSJ, PSS, DES, unpubl. data) and provide counts throughout most of the known ranges of all taxa to determine the geographic structure of ploidy and differences in ploidy among morphologically distinct taxa. We also reconstruct a phylogeny of diploid and polyploid members of the *Humifusa* clade based on nrITS data to investigate the relationship between geographic distribution and evolutionary relationships. We provide counts for another common species in the southeastern U.S., *O. stricta* (Haw.) Haw., 1812, because it has been hypothesized to hybridize with members of the *Humifusa* clade (Benson 1982). In addition, ploidy of the putative hybrid between *O. abjecta* and *O. stricta*, i.e., *O. ochrocentra* Small, 1923, was analyzed. Ploidy determinations of the *Humifusa* clade, coupled with morphological character analysis and further molecular phylogenetics, will aid in the delimitation of species in the group and in determining the origin and evolutionary significance of polyploidy in this clade.

Material and methods

Chromosome counts – Methods follow those of Majure and Ribbens (in press). Briefly, root tips were collected from early morning throughout early afternoon and

placed in 2mM 8-hydroxyquinoline (Soltis 1980) for up to 8 hours at 4°C or in N_2O (Kato 1999) for 1 hour and then fixed in a 3:1 solution of absolute ethanol: glacial acetic acid for 2 to 24 hours. Root tips then were placed in 70% ethanol for at least 2 hours and digested in 40% HCl for 5-10 minutes (depending on the size of the root) at room temperature. Squashes were performed in 60% acetic acid and stained with 1% aceto-orcein dye and viewed on a Zeiss Photomicroscope III (Carl Zeiss, Oberkochen, Germany). To confirm each count, at least three to five metaphase cells were counted per specimen. These multiple counts per sample alleviated concerns regarding endomitosis, which has been reported in the allopolyploid (4*x*), *Opuntia spinosibacca* M.S. Anthony, 1956, (Weedin and Powell 1978), tetraploid *O. pusilla* (Bowden 1945b), as well as in many other angiosperms (e.g., Barrow and Meister 2003, Tate et al. 2009, I. Jordan-Thaden, pers. comm.). We counted chromosomes of 277 individuals of the *Humifusa* clade, 14 individuals of *O. stricta* s.l., three samples of the putative hybrid *O. ochrocentra*, and two individuals of the putative hybrid *O. alta* Griffiths, 1910. Generally, only one accession per population was counted.

Taxonomy – Taxa used for ploidy analysis are listed in Appendix 1. Species delimitations within *O. humifusa* s.l. and *O. macrorhiza* s.l. are problematic, so we recognize both *O. humifusa* and *O. macrorhiza* as broadly circumscribed (Table 1). Thus, we have arranged our counts of plants within these two species (see Appendix 1) according to their various segregates to determine whether the morphological variation of these segregate entities (Table 2) is correlated with cytotype and/or geographical and phylogenetic patterns.

Cytogeographic analysis – We mapped the localities for all of the individuals for which we determined ploidy (277 in number) and incorporated previous counts (n = 41) (Bowden 1945a, Pinkava and McLeod 1971, Pinkava et al. 1973, Weedin and Powell 1978, Pinkava and Parfitt 1982, Pinkava et al. 1985, Weedin et al. 1989, Doyle 1990, Pinkava et al. 1992, Pinkava et al. 1998, Powell and Weedin 2001, Baker et al. 2009a, b, Majure and Ribbens in press) to cover the majority of the geographic distribution of each taxon. This allowed us to explore the geographic boundaries of the different ploidal levels encountered in this clade and construct hypotheses regarding polyploid formation and speciation.

Phylogenetic analysis – We generated sequences from the nuclear ribosomal internal transcribed spacer (nrITS: White et al. 1990) for a sample of diploid (n = 6) and polyploid taxa (n = 8) of the *Humifusa* clade from the eastern and western U.S. (Table 3). *Opuntia basilaris* Engelm. & J.M. Bigelow, 1856, was used as an outgroup based on previous analyses of *Opuntia* (LCM unpubl. data). A phylogenetic analysis of these data was carried out to determine whether the geographic distribution of ploidy (as determined here) was correlated with the evolutionary history of the clade. We carried out a Maximum Likelihood analysis using RAxML (Stamatakis 2006) running 10000 bootstrap pseudoreplicates under 25 rate categories and the GTR+Γ model of molecular evolution.

Table 3. Taxa used in phylogenetic analyses of ITS sequence data given with their GenBank accession numbers.

Accession	Locality	GenBank accession #
Opuntia basilaris (outgroup)	Inyo Co., CA R. Altig s.n.	JF786913
Opuntia abjecta (2*x*)	Monroe Co., FL LCM 3908	JF787021
Opuntia abjecta (4*x*)	Monroe Co., FL LCM 3318	JQ245716
Opuntia ammophila (2*x*)	Marion Co., FL LCM 2826	JF786904
Opuntia austrina (2*x*)	Highlands Co., FL LCM 3450	JF786911
Opuntia cespitosa (4*x*)	Scott Co., MO LCM 2441	JQ245717
Opuntia humifusa (4*x*)	Warren Co., VA LCM 3800	JQ245718
Opuntia lata (2*x*)	Irvin Co., GA LCM 3785	JF786949
Opuntia macrorhiza (4*x*)	Kerr Co., TX LCM 3510	JF786960
Opuntia nemoralis (4*x*)	Garland Co., AR LCM 2196	JQ245720
Opuntia pusilla (2*x*)	Lowndes Co., MS LCM 843	JQ245721
Opuntia pusilla (3*x*)	Baldwin Co., AL LCM 1091	JF786985
Opuntia pusilla (4*x*)	Jackson Co., MS LCM 1920	JF786986
Opuntia tortispina (6*x*)	Hutchinson Co., TX LCM 3533	JF787020
Opuntia xanthoglochia (2*x*)	Bastrop Co., TX LCM 1982	JQ245719

Results

The base chromosome number for Cactaceae has been well established as $x = 11$ (Remski 1954, Pinkava and McLeod 1971, Lewis 1980, Pinkava et al. 1985, Pinkava 2002), and we saw no deviation from this in our counts (Appendix 1). Out of 318 counts of the *Humifusa* clade, including 41 from the literature, 210 (66%) were polyploid and 108 (34%) were diploid. Diploid ($2n = 2x = 22$) and tetraploid ($2n = 4x = 44$) *O. humifusa* s.l. and *O. macrorhiza* s.l. were discovered (Fig. 1A-D, I-J, L). Diploid *O. humifusa* s.l. is restricted entirely to the southeastern U.S., whereas diploid *O. macrorhiza* s.l. is restricted entirely to the southwestern U.S. (eastern Texas (see Appendix 1) and southeastern New Mexico (M. Baker and D.J. Pinkava pers. comm.)). Tetraploid members of *O. humifusa* s.l. and *O. macrorhiza* s.l. are much more widely distributed throughout the U.S. than are their diploid relatives (Fig. 2). Tetraploids of *O. humifusa* s.l. are found from Massachusetts south to the southeastern U.S. where they abut the distribution of diploid taxa and throughout the eastern and midwestern U.S. Tetraploid *O. macrorhiza* s.l. is distributed throughout parts of the Great Plains through the midwestern U.S., most of the southwestern U.S., parts of the Rocky Mountains, and the upper Sierra Madre Occidental in Sonora, Mexico (Fig. 2).

Diploid, triploid, and tetraploid populations of *O. pusilla* were discovered (Fig. 1E-G) throughout its restricted range in the southeastern U.S. (Fig. 3). Interestingly, with the exception of two populations, polyploid individuals (3*x* and 4*x*) were mostly confined to the coastline, although diploid populations were much more widespread throughout the interior part of the distribution of the species (Fig. 3). Of the three examples of *O. abjecta* sampled from the Florida Keys, one was dip-

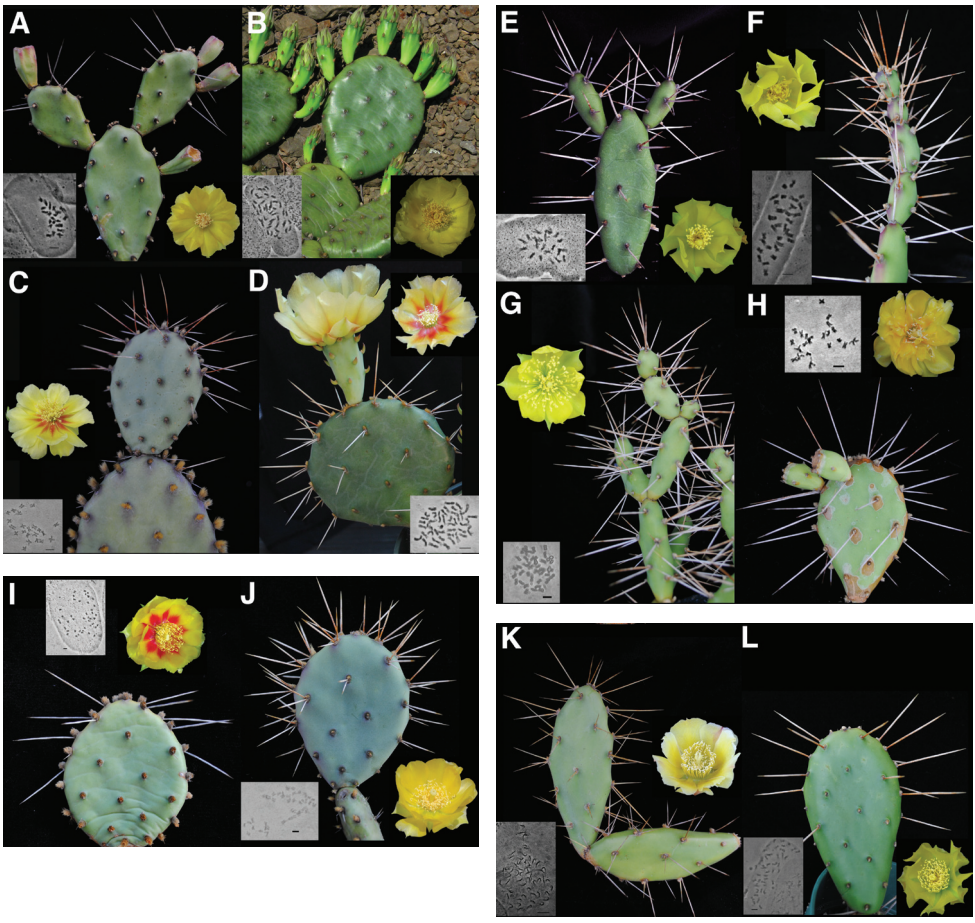

Figure 1. Selected taxa in the *Humifusa* clade with associated chromosome squashes **A** diploid *O. humifusa* (*O. lata*) *LCM 4106* **B** tetraploid *O. humifusa* s.s. *LCM 3810* **C** diploid *O. macrorhiza* (*O. xanthoglochia*) *LCM 1983* **D** tetraploid *O. macrorhiza LCM 3510* **E** diploid *O. pusilla LCM 753* **F** triploid *O. pusilla LCM 1033* **G** tetraploid *O. pusilla LCM 3700* **H** diploid *O. abjecta LCM 3908* **I** tetraploid *O. humifusa (O. cespitosa) LCM 2610* **J** tetraploid *O. humifusa* (*O. nemoralis) LCM 4204* **K** pentaploid *O. ochrocentra LCM 3907* and **L** tetraploid *O. humifusa* (*O. pollardii) LCM 769*. Bars on photomicrographs = 5μm.

loid (Fig. 1H), and two were tetraploid. *Opuntia tortispina* (southwestern U.S.) was hexaploid in six and tetraploid in one of the populations examined (see Fig. 2 for hexaploid distribution).

Individuals of *O. stricta* sampled from the southeastern U.S. were all hexaploid. Samples included members of the taxa considered by some (Anderson 2001) to be *O. dillenii* (Ker-Gawl.) Haw., 1819, and *O. stricta*. Three individuals of the putative hybrid *O. ochrocentra* from two localities in the Florida Keys were pentaploid (Fig. 1K), and the putative hybrid *O. alta* was hexaploid.

Maximum likelihood analysis of ITS data reveals that the *Humifusa* clade is made up of two well-supported subclades. One is restricted to the southeastern U.S. and

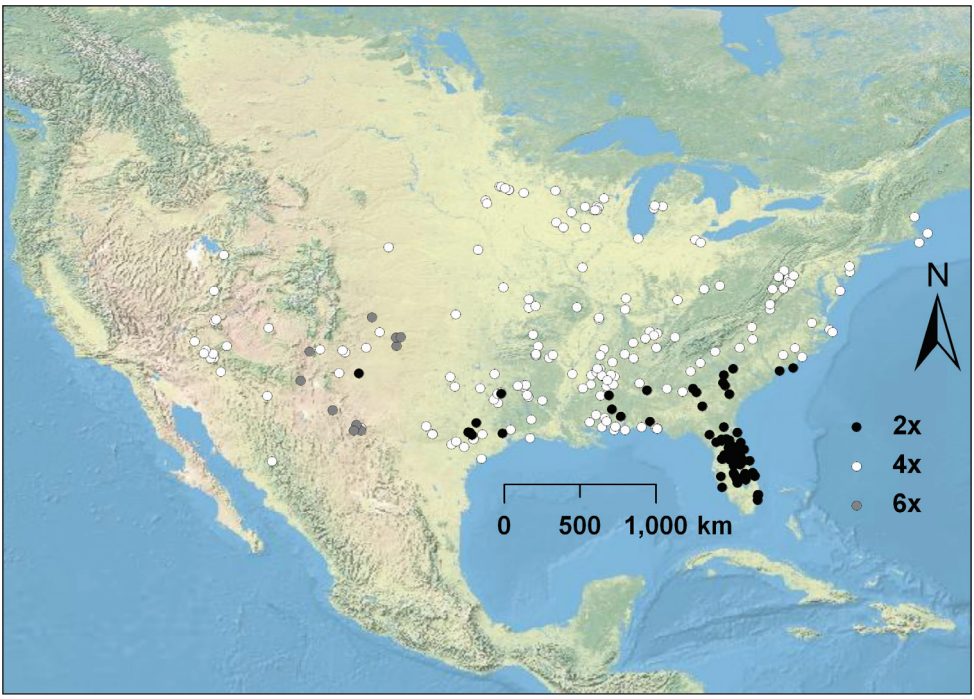

Figure 2. Cytogeography of *O. humifusa* s.l., *O. macrorhiza* s.l., *O. pottsii,* and *O. tortispina*. Diploids are represented with black circles, tetraploids by white circles, and hexaploids are represented by gray circles. *Opuntia humifusa* diploids are confined to the southeastern U.S., and *O. macrorhiza* diploids are located in eastern Texas and southeastern New Mexico.

includes polyploid members of *O. pusilla* and *O. abjecta*, and the other includes southwestern diploid *O. macrorhiza* and all other polyploids pertaining to *O. humifusa* s.l., *O. macrorhiza* s.l., and *O. tortispina*. There is no further resolution within the tree at the species level using ITS (Fig. 4). Species relationships within these two clades are further resolved with the addition of other loci (LCM unpubl. data), however, that is beyond the scope of this study.

Discussion

Opuntia macrorhiza has only been recorded previously as tetraploid (Pinkava et al. 1971, 1973, 1977, 1992, 1998; Powell and Weedin 2001, 2004; Pinkava 2003). These are the first reports of diploid *O. macrorhiza* and likely represent descendants of those progenitors from which tetraploid *O. macrorhiza* s.l. and other polyploids arose. Likewise, this is the first report of diploid and triploid *O. pusilla*, which was formerly known only from tetraploid counts (Bowden 1945a).

Diploid members of *O. humifusa* s.l. (e.g., represented by the segregate taxa *O. ammophila* Small, 1919, *O. austrina* Small, 1903, *O. lata* Small, 1919, in this study;

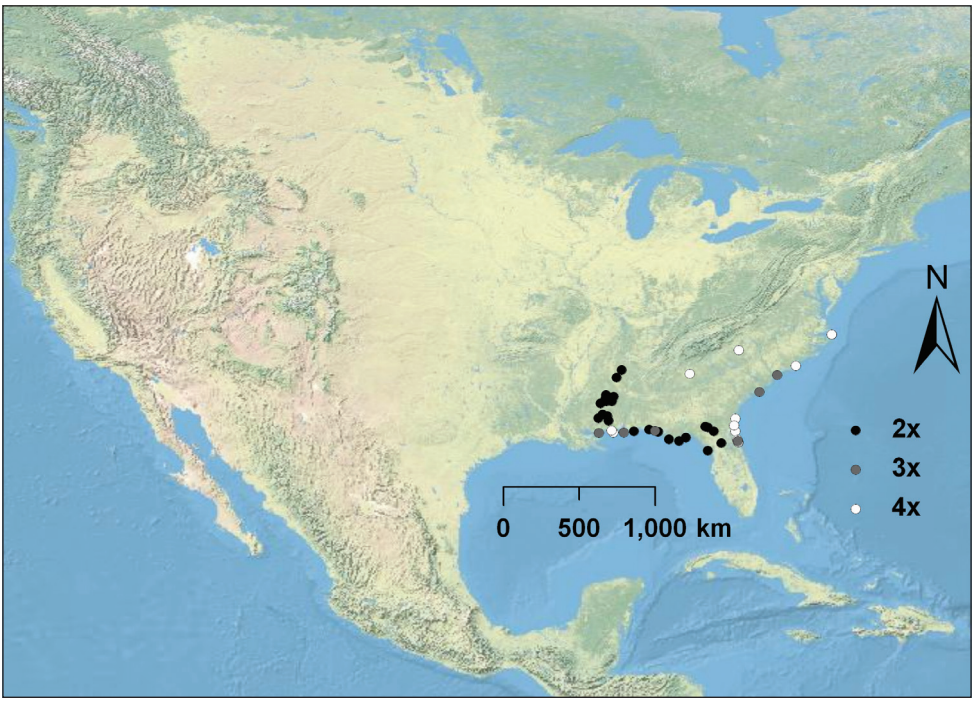

Figure 3. Cytogeography of *O. pusilla*. Diploids are represented by black circles, triploids by gray circles, and tetraploids by white circles. Note that most polyploids are restricted to coastal areas.

see also Appendix 1) exhibit high levels of morphological variability but each is diagnosable morphologically, which suggests that these segregate taxa may need to be recognized at the species level. Likewise, diploid material of *O. macrorhiza* s.l. from eastern Texas (e.g., *O. xanthoglochia* Griffiths, 1910, in this study; see also Appendix 1) and southeastern New Mexico is morphologically distinct from tetraploid material of *O. macrorhiza* s.l., which may also justify the recognition of *O. xanthoglochia* and *O. macrorhiza* as separate species.

Our hexaploid counts of *O. stricta* are consistent with those of Pinkava et al. (1992) and Negrón-Ortiz (2007). In contrast, Spencer (1955) reported *O. stricta* from Puerto Rico to be diploid. Other authors have also found Spencer's counts from Puerto Rico to be inconsistent with more recent counts (e.g., Negrón-Ortiz 2007 for *Consolea* Lem., 1862).

Our three pentaploid counts of *O. ochrocentra* support the proposed hybrid origin of this species between hexaploid *O. stricta* ($2n = 66$) and diploid *O. abjecta* ($2n = 22$) through unreduced gametes of *O. abjecta*. *Opuntia ochrocentra* also exhibits intermediate morphological characters (e.g., growth form, spine characters) that further support its hybrid origin (LCM unpubl. data).

Diploid refugia and polyploid formation – Polyploidy is very common within the *Humifusa* clade, occurring in 66% of the samples reported here. Most researchers that have studied *Opuntia* cytologically have found polyploid taxa (e.g., Bowden 1945a, Weedin and Powell 1978, Pinkava et al. 1985, Doyle 1990, Segura et al. 2007, Baker

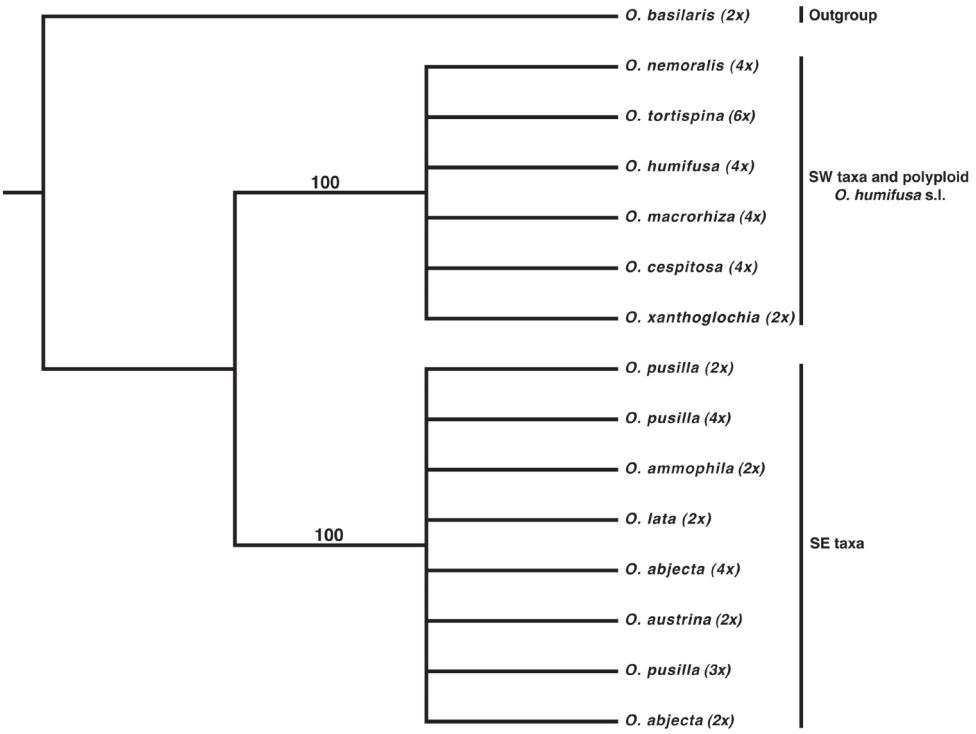

Figure 4. Majority rule consensus topology from 10000 ML bootstrap pseudoreplicates using RAxML based on the nrITS region. The western diploid *O. macrorhiza* s.l. (*O. xanthoglochia*) forms a well-supported clade with polyploid *O. macrorhiza, O. tortispina*, and the eastern polyploid morphotypes of *O. humifusa* s.l. (*O. cespitosa, O. humifusa*, and *O. nemoralis*). The southeastern diploid morphotypes of *O. humifusa* s.l. (*O. ammophila, O. austrina, O. lata*) and diploid *O. abjecta* and *O. pusilla* form a well-supported clade with polyploid members of *O. pusilla* and *O. abjecta*.

et al. 2009a, b, Majure and Ribbens in press, but see Spencer 1955). All diploids in our analysis were restricted to either the southeastern or southwestern (eastern Texas and southeastern New Mexico) U.S., and the polyploid individuals were found nearly everywhere in between as well as north of these two diploid "refugia." The disjunct pattern observed here in the *Humifusa* clade and in other studies between the southeastern U.S. and the southwestern U.S. is thought to have occurred as a result of the disruption of a semi-arid zone along the Gulf Coast region during the mid-Pleistocene (Webb 1990, Althoff and Pellmyr 2002). These two areas likely served as glacial refugia for a variety of animals and plants (e.g., Remington 1968, Davis and Shaw 2001, Al-Rabab'ah and Williams 2002, Althoff and Pellmyr 2002, Soltis et al. 2006, Waltari et al. 2007, Whittemore and Olsen 2011) and may have promoted current species richness and genetic diversity in southern populations (Hewitt 2000). Specifically, Swenson and Howard (2005) identified southeastern Texas and northern Florida as Pleistocene refugia for animal and plant species. Species from these regions subsequently came into contact following the last glacial maximum and formed hybrid zones at contact areas expand-

ing out from these refugia. Swenson and Howard (2005) also hypothesized "post-glacial routes of expansion" from these proposed diploid refugia (e.g., Fig. 1, G & H in Swenson and Howard 2005). Those post-glacial routes and diploid contact zones are consistent with the current distributions of polyploid taxa within *O. humifusa* s.l. and *O. macrorhiza* s.l. The restricted diploid and widespread polyploid distribution pattern has been recorded in many other plants and is a common pattern seen in polyploid complexes (Babcock and Stebbins 1938, Stebbins 1950, 1971, DeWet 1971, Lewis 1980, Grant 1981, Parfitt 1991).

The seemingly disjunct southeastern New Mexico diploid population of *O. macrorhiza* s.l. may represent a mere extension of the eastern Texas diploid refugium, which has since been mostly replaced by polyploid taxa. Alternatively, a diploid extension may still exist but was not detected due to the lack of cytological data for populations from east Texas to southeastern New Mexico (Fig. 2). Diploid taxa of other clades (e.g., *O. polyacantha* Haw. var. *arenaria* (Engelm.) Parfitt, 1819) are coincidentally found near the same region (Pinkava 2002, 2003), however, suggesting that a third diploid refugium, i.e., in southeastern New Mexico-western Texas, may need to be recognized.

Pinkava (2003) suggested that an *O. humifusa-O. macrorhiza-O. pottsii* complex originated along the east coast of the U.S. and spread westward to Arizona, where it came into contact and hybridized with *O. polyacantha* and formed the mostly hexaploid *O. tortispina*. From our data, this scenario is plausible in that *O. tortispina* has morphological characters representative of both *O. polyacantha* and *O. macrorhiza* and is found where populations of diploid and tetraploid *O. macrorhiza* s.l. and diploid *O. polyacantha* come into contact. However, considering the two diploid refugia suggested by our analyses and what is known about the historical biogeography of the southeastern U.S. (e.g., Webb 1990), it is likely that the *Humifusa* clade originated in the southwestern U.S. and adjacent northern Mexico, then dispersed eastward into the southeastern U.S. The arid habitat along the coast of the Gulf of Mexico during the mid-Pliocene to early Pleistocene would have been interrupted during the mid-Pleistocene, creating the disjunction and promoting the genetic divergence among diploid populations we see today (Fig. 4). Taxa from these two diploid refugia would have come back into contact and formed the widely successful polyploids of the Midwest and eastern U.S. (Fig. 5). This scenario is further corroborated by phylogenetic analyses, where eastern U.S. polyploids of *O. humifusa* s.l. are resolved in a clade with the southwestern diploid *O. macrorhiza* (Fig. 4). The lower frequency of diploids encountered in western populations of the *Humifusa* clade also suggest that those diploid populations may be older (see Stebbins 1971, p. 157) than those of the southeastern U.S.; however, this could merely be a bias resulting from more limited sampling of western populations.

The various morphotypes of tetraploid *O. macrorhiza* in the western U.S. likely arose from southwestern diploid populations but subsequently spread in all directions after formation. Tetraploid *O. macrorhiza* appears to have arisen numerous times, given that several morphotypes exist throughout its range. However, only two diploid mor-

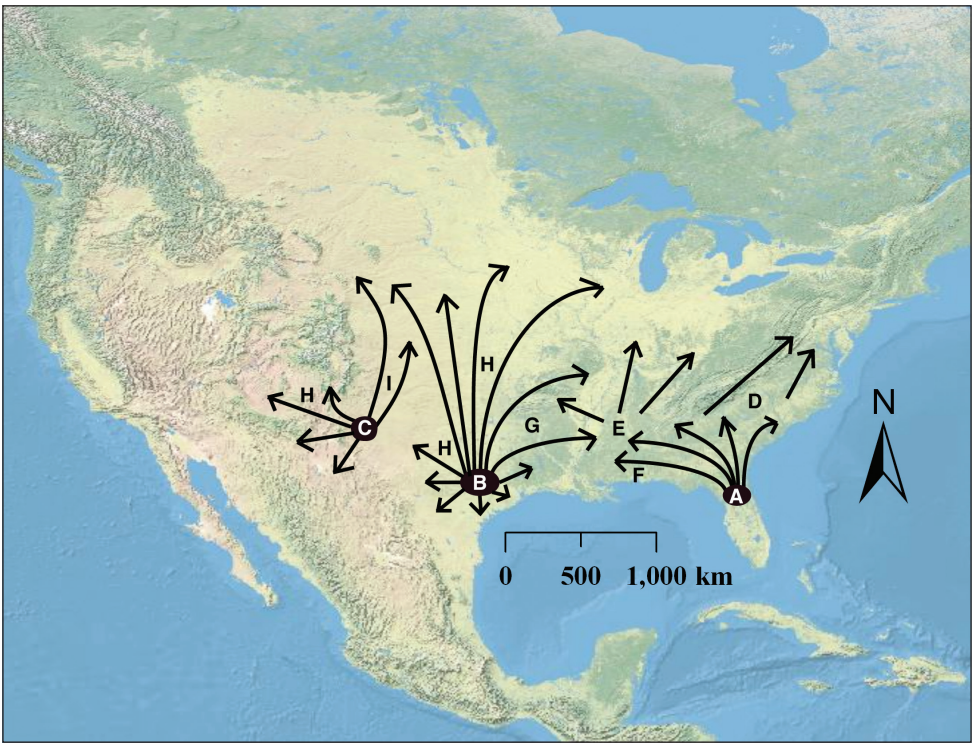

Figure 5. Hypothetical origin and subsequent dispersal of polyploid taxa from diploid refugia. Diploid refugia are represented by **A** southeastern *O. humifusa* s.l. diploids **B–C** eastern Texas and southeastern New Mexico *O. macrorhiza* s.l. diploids **D–I** represent polyploid formation where **D** represents *O. humifusa* **E** represents *O. cespitosa* **F** represents *O. pollardii* **G** represents *O. nemoralis* **H** represents tetraploid *O. macrorhiza* (showing likely multiple formations), and **I** represents tetra- and hexaploid *O. tortispina*.

photypes are known to exist (eastern Texas and southeastern New Mexico), suggesting that other ancestral diploids may have since gone extinct or have not yet been found, or that polyploid taxa exhibiting unique, derived characters were partly responsible for the origin of certain morphotypes, which have no diploid counterparts.

Stebbins (1971) suggested that there are several degrees of maturation of polyploid complex formation (i.e., initial, young, mature, declining, relictual), which may be deduced by comparing the relative geographic distribution of polyploids versus diploids. By these criteria, *Opuntia humifusa* s.l. and *O. macrorhiza* s.l. may represent a mature polyploid complex. The diploid taxa are less common than polyploids and are largely restricted in distribution, whereas the polyploid taxa are much more widespread. Stebbins (1971) also proposed that mature polyploid complexes are relatively young, derived during the Plio- or Pleistocene epochs. This scenario would place polyploid formation in the *Humifusa* clade at the same time as Pleistocene megafauna. Thus, frequent environmental disturbances associated with glacial and interglacial cycles could have mediated the repeated contact of divergent diploid taxa leading to polyploid formation. Migrating herbivores would have then dispersed those polyploid

products over large geographic areas (Jansen 1986). Divergence time estimation of the *Humifusa* clade places the origin of the clade in the late Pliocene to early Pleistocene (LCM, RP, PG, WSJ, PSS, DES unpubl. data), in agreement with this scenario. The occurrence of only polyploid individuals in previously glaciated areas of the U.S. provides further evidence for their subsequent spread into those available niches following the last glacial maximum.

Many polyploid populations of *O. humifusa* s.l. and *O. macrorhiza* s.l., especially in the eastern U.S., are largely isolated from one another and from diploid populations, suggesting that polyploid formation is not ongoing, at least on such a large scale as during the Pleistocene or immediately after the last glacial maximum. In contrast, polyploids in *O. pusilla* are mostly sympatric with diploids in the Gulf of Mexico region and are represented by triploids and tetraploids. Polyploids of *O. pusilla* also do not share the wide geographic distribution of those polyploids derived from *O. humifusa* s.l. and *O. macrorhiza* s.l. These observations suggest that the polyploids of *O. pusilla* may have formed only recently, do not share comparable dispersal agents, or lack the obvious adaptive advantages of those polyploids derived from *O. humifusa* s.l. and *O. macrorhiza* s.l.

Many polyploid populations of *O. humifusa* s.l. and *O. macrorhiza* s.l. occupy northerly distributions and thus have a very high tolerance to cold temperatures. The hexaploid *Opuntia fragilis* (Nutt.) Haw., 1819 (not in the *Humifusa* clade) similarly inhabits areas of northern North America (Parfitt 1991, Loik and Nobel 1993, Ribbens 2008, Majure and Ribbens in press), with diploid relatives (e.g., *O. polyacantha* var. *arenaria*) restricted to the southwestern U.S. (Parfitt 1991, Pinkava 2002). Thus, certain polyploid taxa appear to be more cold-resistant than their southerly diploid relatives (and presumed progenitors). *Opuntia humifusa* s.l. from northern areas of its distribution can withstand temperatures of -20°C (Nobel and Bobich 2002). However, the cold tolerance of diploid taxa has not been tested. Certain polyploid taxa of the *Humifusa* clade may therefore be better adapted to adverse environmental conditions than their diploid progenitors, which may partly explain their wide distribution relative to their diploid counterparts.

Agamospermy – The tetraploid *O. cespitosa* (an entity within *O. humifusa* s.l.; see Table 1) produces viable seed in the absence of outcrossing (Majure pers. obsv.), so this taxon is either self-compatible, which is common in Cactaceae (Rebman and Pinkava 2001), or agamospermous. Agamospermy is commonly associated with polyploidy (Stebbins 1950, DeWet and Stalker 1974, Harlan and DeWet 1975, Lewis 1980, Grant 1981, Whitton et al. 2008) and has been reported in numerous polyploid *Opuntia* species as well (Reyes-Agüero et al. 2006, Felker et al. 2010), including *O. humifusa* s.l. and *O. stricta* (Naumova 1993). Agamospermy would account for the high level of morphological variation observed among polyploid populations, as a result of the maintenance of a specific genotype within a given population through the lack of recombination (DeWet and Stalker 1974). Some agamic complexes also have wider distributions than their diploid progenitors (Babcock and Stebbins 1938, Stebbins 1950), as do certain polyploid taxa in this study.

Autopolyploidy vs. Allopolyploidy – The mechanism by which *Opuntia* polyploids are formed (auto- vs. allopolyploidy) is unclear. Unreduced gametes have frequently been found in meiotic analyses of Cactaceae (e.g., Pinkava et al. 1977, Pinkava and Parfitt 1982, Pinkava et al. 1985). Unreduced gamete formation coupled with interspecific hybridization (allopolyploidy) likely is a major factor in polyploid formation within the genus, given that *Opuntia* is renowned for hybridization (Benson 1982, Grant and Grant 1982, Pinkava 2002, Griffith 2004, LCM, RP, PG, WSJ, PSS, DES unpubl. data). It is probable that unreduced gamete formation within a single species (autopolyploidy) also plays a role in the formation of polyploids. Autopolyploids have been discovered in Cactaceae (Pinkava et al. 1985, Sahley 1996, Hamrick et al. 2002) and may be more common than is suspected.

Opuntia humifusa as currently circumscribed consists of numerous morphological entities, which are either diploid or tetraploid; those populations differing in ploidy are generally geographically well separated from one another. It is evident from our phylogenetic analysis (Fig. 4) that *O. humifusa* is polyphyletic. Considering morphological and genetic data, it is likely that tetraploid *O. humifusa* is of allopolyploid origin. However, the pattern in *O. pusilla* is different, with populations of diploids found in close proximity to populations of triploids and tetraploids (Fig. 3). This evidence, plus morphological similarity among ploidal levels, suggests possible formation of autopolyploids. This same pattern is seen in other autopolyploid taxa (Lewis 1967, Nesom 1983), although there are exceptions to this pattern (Stebbins 1950, Soltis 1984, Husband and Schemske 1998). Molecular phylogenetic analysis (Fig. 4) and morphological characters (LCM, RP, PG, WSJ, PSS, DES unpubl. data; see Fig. 1E-G) of *O. pusilla* also do not support an interspecific hybrid origin for the different ploidal levels herein observed for this species, although more variable molecular markers, cytogenetic work, and more detailed morphological analyses are needed to appropriately address this question.

Morphological correlations with polyploids – Some polyploid taxa in the *Humifusa* clade share morphological characters with diploids and other polyploids, suggesting that they may be derived from hybridization (Table 2). *Opuntia nemoralis* Griffiths, 1913, (Fig. 1J; an entity within *O. humifusa* s.l.; see Table 1) shares spine color and orientation, cladode color, and glochid color of tetraploid *O. macrorhiza* (from Arkansas), although, it possesses small and easily disarticulating cladodes, retrorsely-barbed spines, and the pile forming growth form and yellow flowers of *O. pusilla* (Fig. 1E-G). *Opuntia cespitosa* (Table 1), as mentioned above, exhibits the red-centered flowers, glaucous-gray cladodes, and dark glochids (Fig. 1I) of tetraploid *O. macrorhiza* (Fig. 1D), as well as the spine characters of diploid *O. humifusa* s.l. (= *O. ammophila*, *O. austrina*, *O. lata*; Table 2).

Throughout the distribution of the most common polyploid taxa, there also are polyploid populations that appear to be introgessive products of hybridization with other polyploids. For instance, in Michigan, Wisconsin, and western Illinois, certain populations display characters of both *O. cespitosa* and tetraploid *O. macrorhiza* (see Majure 2010, Fig. 1). In Bibb County, Alabama, populations appear to be interme-

diate between *O. cespitosa* and *O. pollardii* Britton & Rose, 1908, (tetraploids of *O. humifusa* s.l.; see Table 1), with the red-centered flowers and rotund cladodes of *O. cespitosa*, but the yellowish glochids and light green cladode color of *O. pollardii*. In Fayette County, Tennessee, plants appear intermediate between *O. humifusa* s.s. (i.e., tetraploid *O. humifusa* represented by the type collection) and *O. cespitosa*, having the yellowish glochids of tetraploid *O. humifusa* s.s. and the spine characters of *O. cespitosa*. Each one of the areas in which these intermediate plants occur appears to be a region of secondary contact, where polyploid taxa have introgressed to form new polyploid morphotypes that exhibit characters of both of the putative parents.

In the eastern U.S., most populations are represented by only one morphotype and thus appear to be morphologically stable (except for typically variable characters such as spine number; see Rebman and Pinkava 2001), indicating that hybridization is not ongoing among genomically distinct polyploid taxa. In contrast, in central Arkansas and populations farther west, more than one species and/or morphotype may be encountered within a given population. Also, in many coastal populations throughout the southeastern U.S., more than one species may be encountered, and putative hybrid taxa are sometimes observed.

Conclusions

Members of the *Humifusa* clade are found throughout most of the continental U.S., with no obvious breaks or disjunctions in distribution patterns until detailed analyses of chromosome number were carried out. Our analyses indicate that diploid taxa in the *Humifusa* clade are presently confined to the southwestern and the southeastern U.S., which likely represent Pleistocene refugia for these taxa. Polyploid taxa of *O. humifusa* s.l. and *O. macrorhiza* s.l. were likely formed when diploids from these two refugia came into contact during interglacial cycles of the Pleistocene. This scenario is supported further by phylogenetic analyses, in which two clades correspond to these two diploid refugia, and polyploid taxa are found in either clade. Polyploid taxa likely also contributed to the diversity of polyploid morphotypes through secondary contact and introgression with other polyploids. After the end of the last glacial maximum, open niches would have been readily available for colonization by polyploid taxa produced towards the leading edge of the expansion and distribution of the *Humifusa* clade. These polyploids subsequently dispersed throughout most of the continent and occupied all suitable habitats available after glacial retreat, accounting for the distribution that we see today. Distributional success was enabled by the extreme cold tolerance displayed by many of the polyploid taxa, which allowed them to colonize more northern areas presumably unsuitable for diploid taxa.

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Appendix 1

Currently recognized *Opuntia* species investigated are listed (1-6). Synonyms of recognized species (sensu Benson 1982, Pinkava 2003, and Powell et al. 2008 in part; see Table 1) and their respective ploidy are given below the recognized species name. Recognized species are split by ploidy, where species have more than one cytotype. Their somatic chromosome number is given along with locality, collector, and repository according to Index Herbariorum (Thiers 2011). Taxa counted for the first time or cytotypes not previously recorded for a species are delimited with an asterisk (*). All counts were made by L.C. Majure.

1) *Opuntia abjecta* Small

* ***Opuntia abjecta* Small; 2*n* = 22 Florida,** Monroe Co., *LCM 3908* (FLAS). * ***Opuntia abjecta* Small; 2*n* = 44, Florida,** Monroe Co., *LCM 3318* (FLAS), Monroe Co., *KS s.n.* (FLAS).

2) *Opuntia humifusa* (Raf.) Raf.

***Opuntia humifusa* (2*x*) taxa: *Opuntia ammophila* Small; 2*n* = 22, Florida,** Brevard Co., *LCM 2087* (MISSA), Broward Co., *KS 62* (FLAS), Flagler Co., *LCM 3222* (FLAS), Indian River Co., *LCM 4182* (FLAS), Indian River Co., *LCM 4183* (FLAS), Indian River Co., *LCM 4184* (FLAS), Lake Co., *LCM 3246* (FLAS), Lake Co., *LCM 4093* (FLAS), Marion Co., *LCM 2753* (FLAS), Marion Co., *LCM 2754* (FLAS), Marion Co., *LCM 2826* (FLAS), Marion Co., *LCM 3247* (FLAS), Okeechobee Co., *LCM 4185* (FLAS), Okeechobee Co., *LCM 4186* (FLAS), Orange Co., *LCM 2086* (MISSA), Orange Co., *LCM 3962* (FLAS), Osceola Co., *LCM 3702* (FLAS), Osceola Co., *LCM 4181* (FLAS), Osceola Co., *LCM 4189* (FLAS), Putnam Co., *LCM 3248* (FLAS), St. Johns Co., *K.S. s.n.* (FLAS), St. Lucie Co., *LCM 3704* (FLAS), St. Lucie Co., *LCM 3705* (FLAS), St. Lucie Co., *LCM 3708* (FLAS), Seminole Co., *LCM 2085* (MISSA), Volusia Co., *LCM 3224* (FLAS), Volusia Co., *LCM 3232* (FLAS). ***Opuntia austrina* Small; 2*n* = 22, Florida,** Charlotte Co., *KS 45* (FLAS), Highlands Co., FL *KS 64* (FLAS), Highlands Co., *LCM 3450* (FLAS), Highlands Co., *LCM 3975* (FLAS), Highlands Co., *LCM 3976* (FLAS), Highlands Co., *LCM 3978* (FLAS), Okeechobee Co., *KS 29* (FLAS), Okeechobee Co., *KS 42* (FLAS), Palm Beach Co.,

LCM 3970 (FLAS), Palm Beach Co., *LCM 3973* (FLAS), Polk Co., *KS s.n.* (FLAS), Polk Co., *LCM 3979* (FLAS). ***Opuntia lata*** **Small; 2*n* = 22, Alabama,** Autauga Co., *LCM 2043* (MISSA), Mobile Co., *LCM 4194* (FLAS), **Florida,** Alachua Co., *LCM 3991* (FLAS), Alachua Co., *LCM 4061* (FLAS), Alachua Co., *LCM 4064* (FLAS), Hernando Co., *LCM 3948* (FLAS), Highlands Co., *LCM 3977* (FLAS), Lafayette Co., *LCM 2795* (FLAS), Lake Co., *KS 15* (FLAS), Lake Co., *LCM 4117* (FLAS), Levy Co., *LCM 3645* (FLAS), Manatee Co., *LCM 4065* (FLAS), Okaloosa Co., *LCM 3954* (FLAS), Okeechobee Co., *LCM 4187* (FLAS), Okeechobee Co., *LCM 4188* (FLAS), Orange Co., *LCM 4174* (FLAS), Palm Beach Co., *LCM 3971* (FLAS), Putnam Co., *LCM 4106* (FLAS), Sumter Co., *LCM 3238* (FLAS), Sumter Co., *LCM 4066* (FLAS), **Georgia,** Charlton Co., *LCM 4190* (FLAS), Crawford Co., *JH s.n.* (FLAS), Irwin Co., *LCM 3785* (FLAS), Perry Co., *LCM 3786* (FLAS), Tatnall Co., *JH s.n.* (FLAS), **Mississippi,** Newton Co., *LCM 938* (MISSA), Wayne Co., *LCM 1290* (MISSA), **South Carolina,** Aiken Co., *LCM 3588* (FLAS), Horry Co., *LCM 3832* (FLAS).

Opuntia humifusa **(4*x*) taxa: ****Opuntia allairei*** **Griffiths; 2*n* = 44, Texas,** Liberty Co., *LCM 3504* (FLAS). ****Opuntia cespitosa*** **Raf.; 2*n* = 44, Alabama,** Bibb Co., *LCM 2042* (MISSA), Colbert Co., *LCM 2610* (MISSA), Lawrence Co., *LCM 2609* (MISSA), **Arkansas,** Garland Co., *LCM 2198* (FLAS), Garland Co., *LCM 4203* (FLAS), Garland Co., *LCM 4205* (FLAS), Saline Co., *LCM 2194* (MISSA), Yell Co., GPJ s.n. (FLAS), **Illinois,** Cass Co., IL *ER s.n.* (FLAS), Jo Daviess Co., IL *ER s.n.* (FLAS), **Kentucky,** Anderson Co., *LCM 3276* (FLAS), **Louisiana,** Caddo Parish, *LCM 4200* (FLAS), Caddo Parish, *LCM 4201* (FLAS), Caddo Parish, *LCM 4202* (FLAS), **Massachusetts,** Dukes Co., *BC s.n.* (FLAS), **Mississippi**, Lee Co., MS *JH s.n.* (FLAS), Lowndes Co., *LCM 755* (MISSA), Oktibbeha Co., *LCM 1380* (MISSA), Scott Co., *LCM 2563* (MISSA), **Tennessee,** Bledsoe Co., *LCM 1938* (MISSA), Cannon Co., *LCM 2072* (MISSA), Davidson Co., *JH s.n.* (FLAS), Fayette Co., *LCM 1956* (MISSA; note *O.* cf. *cespitosa*), Fayette Co., *JH s.n.* (note *O.* cf. *cespitosa* FLAS), Franklin Co., *BLS 2061* (FLAS), Lewis Co., *JH s.n.* (FLAS), Marshall Co., *JH s.n.* (FLAS), Rutherford Co., *JH s.n.* (FLAS), **Texas,** Lamar Co., *BS 2069* (FLAS), **Virginia,** Fredrick Co., *LCM 3806* (FLAS). ***Opuntia humifusa*** **(Raf.) Raf.; 2*n* = 44, Alabama,** Marion Co., AL *JH s.n.* (FLAS), **Delaware,** Sussex Co., *LCM 3824* (FLAS), **Georgia**, Dekalb Co., GA *LCM 3787* (FLAS), Jackson Co., *LCM 3789* (FLAS), Marion Co., *JH s.n.* (FLAS), **Maryland,** Alleghany Co., *LCM 3810* (FLAS), **Massachusetts**, Barnstable Co., MA *LCM 3814* (FLAS), **Mississippi,** Calhoun Co., MS *JH s.n.* (FLAS), Carroll Co, *LCM 799* (MISSA), Choctaw Co., *KP 499* (MMNS), Grenada Co., *LCM 1833* (MISSA), Marion Co., *JH s. n.* (FLAS), Marshall Co., *LCM 1293* (MISSA), Montgomery Co., *LCM 768* (MISSA), Stone Co., *TM s.n.* (FLAS), Webster Co., *KP 498* (MMNS), Yalobusha Co., *LCM 767* (MISSA), **New Hampshire,** Rockingham Co., *BN s.n.* (FLAS), **New Jersey,** Atlantic Co., *VD s.n.* (FLAS), Burlington Co., *LCM 3821* (FLAS), **North Carolina,** Bladen Co., *JH s.n.* (FLAS), Currituck Co., *LCM 3825* (FLAS), Dare Co., *LCM 3827* (FLAS), Onslow Co., *LCM 3829* (FLAS), Rowan Co., *LCM 3793* (FLAS), Surry Co., *JH s.n.* (FLAS), **South Carolina,** Pickens Co., *LCM 3790* (FLAS), York Co., *LCM 3791* (FLAS), **Virginia,** Fredrick Co., *LCM 3807* (FLAS), Page Co.,

LCM 3799 (FLAS), Warren Co., *LCM 3800* (FLAS), **West Virginia,** Hampshire Co., *LCM 3808* (FLAS), Mineral Co., *LCM 3809* (FLAS), Pendleton Co., *ER s.n.* (FLAS). ****Opuntia nemoralis* Griffiths, 2*n* = 44, Arkansas,** Garland Co., *LCM 2192* (MISSA), Garland Co., *LCM 2196* (MISSA), Garland Co., *LCM 4204* (FLAS); **Louisiana,** Beauregard Parish, *CR s.n.* (FLAS), Cameron Parish, *LCM 4196* (FLAS), DeSoto Parish, *LCM 4198* (FLAS), Red River Parish, *LCM 4199* (FLAS), Winn Parish, *BLS 2053* (FLAS). ****Opuntia* cf. *nemoralis* Griffiths, 2*n* = 44, Arkansas,** Pulaski Co., *BLS 2131* (FLAS), Yell Co., TW s.n. (FLAS). ****Opuntia pollardii* Britton & Rose; 2*n* = 44, Alabama,** Baldwin Co., *LCM 1082* (MISSA), **Florida,** Santa Rosa Co., *LCM 1075* (MISSA), Walton Co., *LCM 1067* (MISSA), Walton Co., *LCM 1070* (MISSA), **Louisiana,** Washington Parish, *CR s.n.* (FLAS), **Mississippi,** Forrest Co., *LCM 806* (MISSA), Hancock Co., *LCM 748* (MISSA), Jackson Co., *LCM 1921* (MISSA), Jackson Co., *LCM 1297* (MISSA), Jackson Co., *LCM 4057* (FLAS), Jackson Co., *LCM s.n.* (MMNS), Neshoba Co., *LCM 1201* (MISSA), Noxubee Co., *LCM 1156* (MISSA), Stone Co., *TM s.n.* (FLAS), Winston Co., *LCM 769* (MISSA).

3) *Opuntia macrorhiza* Engelm.

***Opuntia macrorhiza* (2x) taxa: * *Opuntia xanthoglochia* Griffiths, 2*n* = 22, Texas,** Bastrop Co., *LCM 1982* (MISSA), Bastrop Co., *MJM 949* (FLAS), Fayette Co., *LCM 1983* (MISSA), Harris Co., *BLS 2089* (FLAS), Milam Co., TX *MJM 947* (FLAS), Smith Co., *BLS 2082* (FLAS).

***Opuntia macrorhiza* (4*x*) taxa: ****Opuntia fusco-atra* Engelm.; 2*n* = 44, Texas,** Fayette Co., *LCM 3505* (FLAS). ****Opuntia grandiflora* Engelm.; 2*n* = 44, Arkansas,** Miller Co., *BLS 2062* (FLAS), **Mississippi,** Bolivar Co., *LCM 1680* (MISSA), Holmes Co., *HS s.n.* (FLAS), Yazoo Co., *LCM 2366* (MISSA), **Texas,** Anderson Co., *BLS 2077* (FLAS), Austin Co., *BLS 2091* (FLAS), Henderson Co., *BLS 2081* (FLAS), Jack Co., *LCM 3536* (FLAS), Leon Co., *BLS 2074* (FLAS), Marion Co., *BLS 2086* (FLAS), Smith Co., *LCM 3540* (FLAS), Van Zandt Co., *BLS 2083* (FLAS). ***Opuntia macrorhiza* Engelm., 2*n* = 44, Arkansas,** Nevada Co., *BLS 2130* (FLAS), Newton Co., *MC s.n.* (FLAS), Pulaski Co., *LCM 4206* (FLAS), **Arizona,** Coconino, *TH s.n.* (FLAS), Coconino, *BW s.n.* (FLAS), **Nebraska,** Keith Co., NE *ER s.n.* (FLAS), Lancaster Co., *TH s.n.* (FLAS), **New Mexico,** Torrance Co., *LCM 3530* (FLAS), **Texas,** Calhoun Co., TX *MJM 962* (FLAS), Dallas Co., *LCM 3539* (FLAS), Gonzales Co., *MJM 958* (FLAS), Kimble Co., *LCM 3511* (FLAS), Kerr Co., *LCM 3508* (FLAS), Kerr Co., *LCM 3510* (FLAS), Palo Pinto Co., *LCM 3537* (FLAS), **Utah,** Salt Lake Co., *TH s.n.* (FLAS), Sevier Co., *TH s.n.* (FLAS).

4) *Opuntia pusilla* (Haw.) Haw.

* ***Opuntia pusilla*, 2*n* = 22, Alabama,** Lamar Co., *JH s.n.* (FLAS), **Florida,** Alachua Co., *LCM 4003* (FLAS), Bay Co., *KS 307* (FLAS), Bay Co., *KS 309* (FLAS), Columbia Co., *LCM 4191* (FLAS), Escambia Co., *KS 328* (FLAS), Franklin Co., *KS 301* (FLAS), Franklin Co., *KS 330* (FLAS), Gulf Co., *KS 325* (FLAS), Hamilton Co., *LCM 4192* (FLAS), Hamilton Co., FL *LCM 4193* (FLAS), Levy Co., *LCM 2819*

(FLAS), **Mississippi,** Clarke Co., *LCM 1270* (MISSA), Forrest Co., *LCM 756* (MISSA), Jasper Co., *LCM 766* (MISSA), Lamar Co., *LCM 1548* (MISSA), Lauderdale Co., *LCM 2094* (MISSA), Lauderdale Co., *LCM 3919* (MISSA), Lowndes Co., *LCM 843* (MISSA), Newton Co., *LCM 828* (MISSA), Newton Co., *LCM 937* (MISSA), Newton Co., *LCM 4211* (FLAS), Perry Co., *LCM 757* (MISSA), Smith Co., *LCM 753* (MISSA), Wayne Co., *TM s.n.* (FLAS), Wayne Co., *TM s.n.* (FLAS). * ***Opuntia pusilla*, 2*n* = 33, Alabama,** Baldwin Co., *LCM 1091* (MISSA), **Florida,** Flagler Co., *LCM 3221* (FLAS), St. Johns Co., *LCM 3219* (FLAS), Walton Co., *LCM 1066* (MISSA), **Mississippi,** Hancock Co., *LCM 1033* (MISSA), **South Carolina,** Horry Co., *JH s.n.* (FLAS), Horry Co., *LCM 3833* (FLAS). ***Opuntia pusilla*, 2*n* = 44, Florida,** Duval Co., *LCM 3700* (FLAS), Nassau Co., *CJ s.n.* (FLAS), St. Johns Co., *LCM 3218* (FLAS), St. John's Co., *KS 9.4.10* (FLAS), **Georgia,** Dekalb Co., *LCM 3788* (FLAS), Glynn Co., *TM s.n.* (FLAS), **Mississippi,** Jackson Co., *LCM 955* (MISSA), Jackson Co., *LCM 1920* (MISSA), **North Carolina,** Dare Co., *LCM 3828* (FLAS), Dare Co., *LCM 3836* (FLAS), New Hanover Co., *LCM 3830* (FLAS), **South Carolina,** York Co., *LCM 3792* (FLAS).

5a) *Opuntia stricta* (Haw.) Haw.

***Opuntia dillenii* (Ker-Gawl.) Haw., 2*n* = 66, Florida,** Charlotte Co., *LCM 3949* (FLAS), Flagler Co., *LCM 3220* (FLAS), Monroe Co., *LCM 3319* (FLAS), Hillsborough Co., *LCM 3952* (FLAS), **Puerto Rico,** Cabo Rojo, *LCM 3843* (FLAS). ***Opuntia stricta* (Haw.) Haw., 2*n* = 66, Alabama,** Mobile Co., *LCM 823* (MISSA), **Florida,** Clay Co., *LCM 3701* (FLAS), Levy Co., *LCM 2820* (FLAS), Monroe Co., *LCM 3320* (FLAS), St. Johns Co., *LCM 3217* (FLAS), Seminole Co., *LCM 2083* (MISSA), **Mississippi,** Jackson Co., *LCM 1922* (MISSA).

5b) Putative hybrids involving *Opuntia stricta*.

***Opuntia alta* Griffiths 2*n* = 66, Louisiana,** Cameron Parish, *LCM 4195* (FLAS), LaFourche Parish, *CR s.n.* (FLAS). * ***Opuntia ochrocentra* Small, 2*n* = 55, Florida,** Monroe Co., *LCM 3907* (FLAS), Monroe Co., *LCM 3968* (FLAS), Monroe Co., *LCM 3969* (FLAS).

6) *Opuntia tortispina* Engelm. & J.M. Bigelow.

***Opuntia tortispina*, 2*n* = 44, New Mexico,** Quay Co., *LCM 3531* (FLAS), ***Opuntia tortispina*, 2*n* = 66, New Mexico,** Benalillo Co., *LCM 3528* (FLAS), Sierra Co., *LCM 3521* (FLAS), **Oklahoma,** Cimarron Co., *ER s.n.* (FLAS), **Texas,** Carson Co., *LCM 3532* (FLAS), Hutchinson Co., *LCM 3533* (FLAS), Hutchinson Co., *LCM 3535* (FLAS).

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Comparative cytogenetic study on two species of the genus *Entedon* Dalman, 1820 (Hymenoptera, Eulophidae) using DNA-binding fluorochromes and molecular and immunofluorescent markers

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Abstract

Karyotypes of *Entedon cionobius* Thomson, 1878 and *E. cioni* Thomson, 1878 (Hymenoptera: Eulophidae) were studied using DNA-binding ligands with different base specificity (propidium iodide, chromomycin A_3, methyl green and DAPI; all these ligands, except for the last one, were used for the first time in parasitic wasps), C-banding, fluorescence *in situ* hybridization (FISH) with a 45S rDNA probe and 5-methylcytosine immunodetection. Female karyotypes of both species contain five pairs of relatively large metacentric chromosomes and a pair of smaller acrocentric chromosomes (2n = 12). As in many other Hymenoptera, males of both *Entedon* Dalman, 1820 species have haploid chromosome sets (n = 6). Fluorochrome staining revealed chromosome-specific banding patterns that were similar between the different fluorochromes, except for the CMA_3- and PI-positive and DAPI-negative band in the pericentromeric regions of the long arms of both acrocentric chromosomes. The obtained banding patterns were virtually identical in both species and allowed for the identification of each individual chromosome. C-banding revealed a pattern similar to DAPI staining, although centromeric and telomeric regions were stained more intensively using the former technique. FISH detected a single rDNA site in the same position on the acrocentric chromosomes as the bright CMA_3-positive band. Immunodetection of 5-methylcytosine that was performed for the first time in the order Hymenoptera revealed 5-methylcytosine-rich sites in the telomeric, centromeric and certain interstitial regions of most of the chromosomes.

Keywords
Hymenoptera, Eulophidae, *Entedon*, chromosomes, karyotypes, base-specific fluorochromes, fluorescence *in situ* hybridization (FISH), DNA methylation

Introduction

Parasitic wasps are a very diverse, taxonomically complicated and economically important group of insects (Rasnitsyn 1980, Heraty et al. 2011). They attack many insect species, including important agriculture and forestry pests. Parasitic Hymenoptera are therefore widely used in both outdoor and indoor biological plant protection (Quicke 1997, Heraty 2004). The estimated number of potentially described species of parasitic Hymenoptera far exceeds 600,000 (Heraty 2009) or even approaches one million (Quicke 1997), but chromosomal analysis has only been performed on approximately four hundred species (Gokhman 2009). Males of parasitic wasps are usually haploid and females are usually diploid (Crozier 1975). The haploid chromosome number varies from 3 to 23 (Gokhman 2009). Nevertheless, karyotypes of most species of parasitic Hymenoptera have only been studied using routine chromosomal staining. Localization of nucleolus organizing regions (NORs) has been determined using AgNOR staining in only a few species of the superfamily Chalcidoidea (e.g. Reed 1993, Bernardo et al. 2008, Gebiola et al. 2012). However, certain base-specific fluorochromes, such as 4', 6-diamidino-2-phenylindole (DAPI) and chromomycin A_3 (CMA_3), are now widely used to visualize chromosome segments with different AT/GC base compositions in many other insect groups, including aculeate Hymenoptera (Rocha et al. 2002, Kuznetsova et al. 2003, Lachowska 2008, Monti et al. 2010). Surprisingly, parasitic Hymenoptera have never been studied using these techniques. Additionally, fluorescence *in situ* hybridization (FISH) with rDNA probes is also a well-known and highly specific method of revealing nucleolus organizers in many organisms (as demonstrated in Winterfeld and Röser 2007, and Cabrero and Camacho 2008). However, *Trichogramma kaykai* Pinto et Stouthamer, 1997 (Trichogrammatidae) remains the only parasitic wasp species that has been studied using this technique (Van Vugt et al. 2005, 2009).

Currently, it is becoming obvious that DNA methylation plays a crucial role in the regulation of gene activity (Field et al. 2004, Vanyushin 2006, Gehring and Henikoff 2007, Suzuki and Bird 2008). Moreover, the first results of full genomic sequencing of three parasitic Hymenoptera species of the genus *Nasonia* Ashmead, 1904 (Pteromalidae) demonstrated that these parasitic wasps carried genes encoding a full DNA methylation toolkit, including all three types of DNA cytosine-5-methyltransferases (The *Nasonia* Genome Working Group 2010). Nevertheless, DNA methylation patterns of chromosomes have never been studied for these parasitic Hymenoptera, or any other parasitic wasp.

The present study used molecular cytogenetic techniques to examine two closely related species of parasitic Hymenoptera that belong to the genus *Entedon* Dalman, 1820 of the family Eulophidae. This genus comprises about 170 described species (http://www.nhm.ac.uk/research-curation/research/projects/chalcidoids/database/index.dsml, on February 8, 2012); however, critical revision of the group is needed. Both studied species, namely, *Entedon cionobius* Thomson, 1878 and *E. cioni* Thomson, 1878, though being slightly habitually different, belong to the same species group, *Entedon cioni*, and attack larvae of the genus *Cionus* Clairville, 1798 (Coleoptera: Curculionidae) in Europe (Gumovsky 1997; see below). Currently, karyotypes of approximately 60 species of the family Eulophidae have been studied (Gokhman 2002, 2009, Gebiola et al. 2012). Hitherto, only the chromosomes of undetermined species of the genus *Entedon* were examined recently (Gokhman 2004).

Materials and methods

Origin of parasitic wasps

Both *Entedon cionobius* and *E. cioni* are gregarious endoparasitoids of beetle larvae of the genus *Cionus*. Host larvae potentially containing broods of *Entedon* species were collected on different plants of the genus *Scrophularia* Linnaeus, 1753 (Lamiales: Scrophulariaceae) in Kiev, Ukraine, in May 2010. Weevil larvae were fed with leaves of the host plant in the laboratory until the emergence of the mature parasitoid larvae. For every brood, a few larvae were retained for identification purposes. Larvae that were reared to adults were subsequently identified by A.V. Gumovsky. Voucher specimens were deposited in the collection of the Institute of Zoology of the National Academy of Sciences of Ukraine.

Preparation of chromosomes

Chromosomal preparations were obtained from cerebral ganglia of prepupae generally following the protocol developed by Imai et al. (1988). Ganglia were extracted from insects dissected in 0.5% (not 1% as proposed by Imai et al. 1988) hypotonic sodium citrate solution containing 0.005% colchicine. The extracted ganglia were then transferred to a fresh portion of hypotonic solution and incubated for 20 min at room temperature. The material was transferred onto a pre-cleaned microscope slide using a Pasteur pipette and then gently flushed with Fixative I (glacial acetic acid: absolute ethanol: distilled water 3:3:4). The tissues were disrupted using dissecting needles in an additional drop of Fixative I. Another drop of Fixative II (glacial acetic acid: absolute ethanol 1:1) was applied to the center of the area, and the more aqueous phase was blotted off the edges of the slide. The same procedure was performed with Fixative

III (glacial acetic acid). The slides were then dried for approximately half an hour and stored for about eight months at -20°C.

Fluorochrome staining

Chromosome spreads were stained with combinations of different fluorochromes, including CMA_3/DAPI and methyl green (MG)/CMA_3 (Schweizer and Ambros 1994), propidium iodide (PI)/DAPI (Kim et al. 2002) and MG/DAPI; the latter technique is analogous to distamycin A (DA)/DAPI staining (Donlon and Magenis 1983).

CMA_3/DAPI staining. The slide was flooded with chromomycin staining solution (0.5 mg/ml in McIlvaine's buffer (pH 7.0) containing 5 mM $MgCl_2$), covered with a coverslip, and incubated at room temperature in the dark overnight. The coverslip was then removed, and the slide was briefly rinsed with distilled water and air-dried. The slide was then flooded with DAPI solution (1 μg/ml in McIlvaine's buffer), covered with a coverslip, and stained in the dark at room temperature for 15 min. The coverslip was then removed, and the slide was briefly rinsed with distilled water before being air-dried. The preparation was then mounted in a 1:l mixture of glycerol/McIlvaine's buffer containing 2.5 mM $MgCl_2$ and sealed with rubber cement. The slide was aged prior to examination by storing in the dark at 30–37°C for a minimum of one day.

MG/CMA_3 staining. The slide was stained with CMA_3 solution (see above) for approximately one hour and then briefly rinsed with distilled water and air-dried. The preparation was then counterstained for l0–20 min with MG solution in McIlvaine's buffer (3.5 μg/ml), briefly rinsed with distilled water, and then air-dried. The slide was then mounted in glycerol and sealed with rubber cement. The preparation was stored for one day at 37°C prior to analysis by epifluorescence microscopy.

PI/DAPI staining. The slide was stained with a PI and DAPI mixture (1 mg/ml and 0.5 mg/ml, respectively, in McIlvaine's buffer) for 20 min with 10 min of pre- and post-incubation in McIlvaine's buffer. The slide was then briefly rinsed with distilled water, air-dried and mounted in VECTASHIELD anti-fading medium (Vector Laboratories).

MG/DAPI staining. The slide was stained with MG solution in McIlvaine's buffer (0.35 mg/ml) for 15–30 min and rinsed with distilled water and air-dried. The preparation was then stained with DAPI solution (see above) for 5 min in the dark, rinsed with distilled water, and then air-dried. The slide was then mounted in a mixture of glycerol and McIlvaine's buffer (1:1).

Fluorescence *in situ* hybridization (FISH)

Plasmid pTa 71 containing the full DNA sequence of the 45S rRNA gene of wheat was used as the probe for visualizing ribosomal genes (Gerlach and Bedbrook 1979). This probe was labeled using Biotin-Nick Translation Mix (Roche). FISH with rDNA

probes was carried out as described previously (Muravenko et al. 2009). Chromosome slides were pretreated with 1 mg/ml RNAse A (Roche) in 2 × SSC at 37°C for 1 h, washed three times for 10 min in 2 × SSC, dehydrated in a series of 70%, 85% and 96% ethanol solutions, and then air-dried. The hybridization mixture contained 50% de-ionized formamide, 10% dextran sulfate, 1% Tween 20 and 2 × SSC. Fifteen microliters of hybridization mixture containing 40 ng of biotin-labeled DNA probe was added to each slide. The slides were then coverslipped, sealed with rubber cement and denatured at 74°C for 5 min. The hybridization was carried out in a moisture chamber at 37°C overnight. After removing the coverslips, the slides were washed twice with 0.1 × SSC at 44°C for 10 min, followed by washing with 2 × SSC at 44°C for 2 × 5 min and with 2 × SSC at room temperature for 5 min. Biotin was detected using avidin-fluorescein isothiocyanate (avidin-FITC) (Vector Laboratories). The slides were mounted in VECTASHIELD anti-fading medium (Vector Laboratories) containing 1.5 μl of DAPI (Sigma-Aldrich).

C-banding

C-banding was carried out as described by Bolsheva et al. (1984), with some modifications. The slides were incubated in a saturated solution of $Ba(OH)_2$ for 6.5 min at room temperature, rinsed in 1 N HCl for 30 sec and then washed with tap water for 15 min. Slides were then incubated in 2 × SSC at 60°C for 1 h, briefly rinsed in distilled water, air-dried, and then stained with 1.5% Giemsa solution (Merck) in 0.075 M phosphate buffer (pH = 6.8) for 1–3 min under control of a light microscope.

Study of DNA methylation patterns

5-methylcytosine was detected according to the protocol described by Pendina et al. (2005), with a few modifications. Chromosomal preparations were denatured in a 2 M HCl solution at 37°C for 20 min. The preparations were washed twice with ice-cold phosphate-buffered saline (1 × PBS) for 1–2 min and then twice with distilled water, before being air-dried. Then, 100 μl of blocking solution containing 1 × PBS, 5% bovine serum albumin and 0.05% Tween 20 was applied under the coverslips to chromosomal preparations. The preparations were then incubated for 40 min in a moisture chamber at 37°C. After incubation, the slides were quickly rinsed with 1 × PBS containing 0.05% Tween 20. Unconjugated mouse anti-5-methylcytosine monoclonal antibody (American Research Products, Inc.) was dissolved in blocking solution (1:200), and 100 μl was applied to the preparations under the 24 × 60 mm coverslips. The preparations were incubated for 60 min in a moisture chamber at 37°C and then rinsed three times with 1 × PBS containing 0.05% Tween 20 at 43°C for 3 min each. Goat anti-mouse IgG Texas Red-conjugated antibody (Santa Cruz Biotechnology, Inc.) was dissolved in blocking solution (1:200), and 100 μl was applied to

the moist preparations under the 24 × 60 mm coverslips. The preparations were then incubated for 40 min in a moisture chamber at 37°C before being rinsed three times with 1 × PBS containing 0.05% Tween 20 at 43°C for 3 min each. The preparations were then quickly rinsed in 1 × PBS, rinsed twice in distilled water, air-dried and then mounted in VECTASHIELD anti-fading medium (Vector Laboratories) containing 1.5 μl of DAPI (Sigma-Aldrich).

Chromosomal analysis

Metaphase plates were studied and photographed using an Olympus BX-61 epifluorescence microscope fitted with Cool Snap Ropper Scientific black-and-white CCD camera. The obtained images were processed using the VideoTesT-Kario 1.5 software (Ista-VideoTesT, Russia).

Results

The overall chromosomal morphology of both *Entedon cionobius* and *E. cioni* was very similar. Diploid female karyotypes of these parasitic wasps were comprised of five pairs of relatively large metacentric chromosomes (approximately 10 μm in length) and a pair of smaller acrocentric chromosomes (approximately 5 μm) (2n = 12). Males had haploid chromosome sets (n = 6). The DAPI banding patterns in *Entedon cionobius* and *E. cioni* were virtually identical. From those patterns, we were able to identify all of the chromosomes within the karyotypes. The DAPI banding patterns demonstrated intensive staining of the pericentromeric regions together with a few weaker interstitial bands on all of the metacentric chromosomes. Additionally, the short arms of the acrocentric chromosomes were also intensively stained (Fig. 1).

A similar banding pattern was observed when using PI and CMA_3 staining. However, there were narrow but distinct pericentromeric DAPI-negative but PI- and

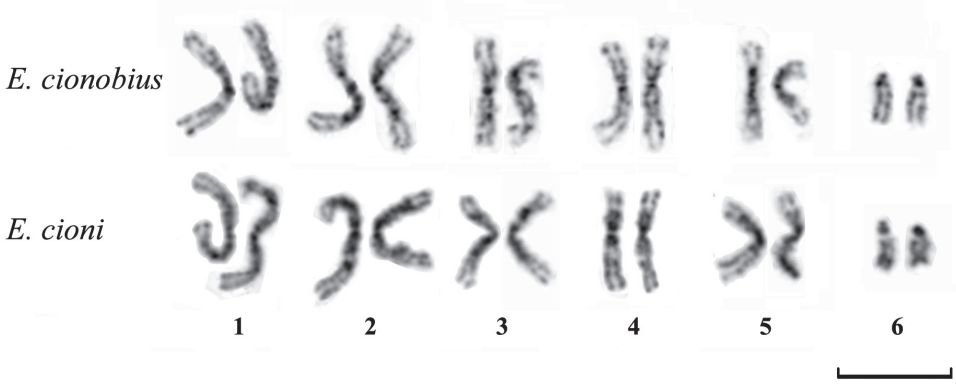
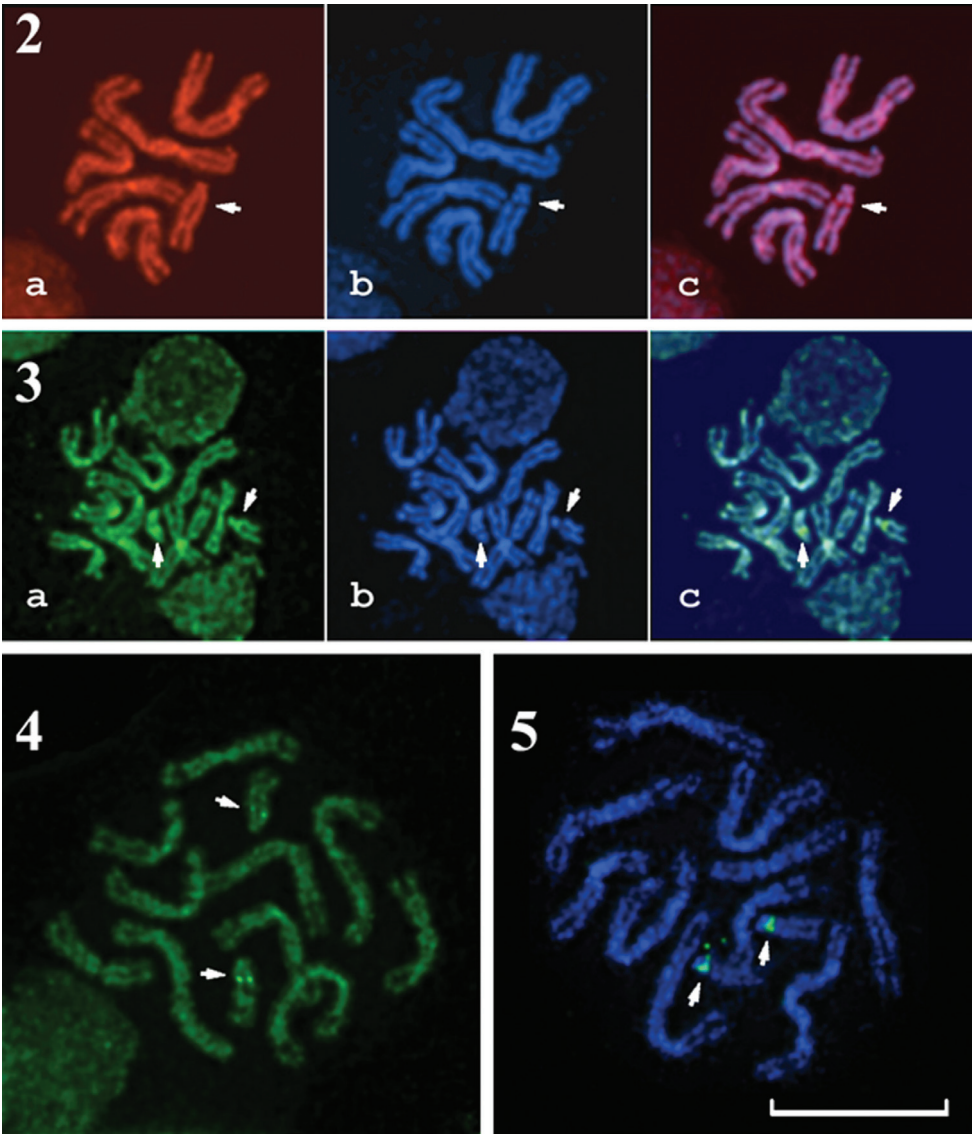
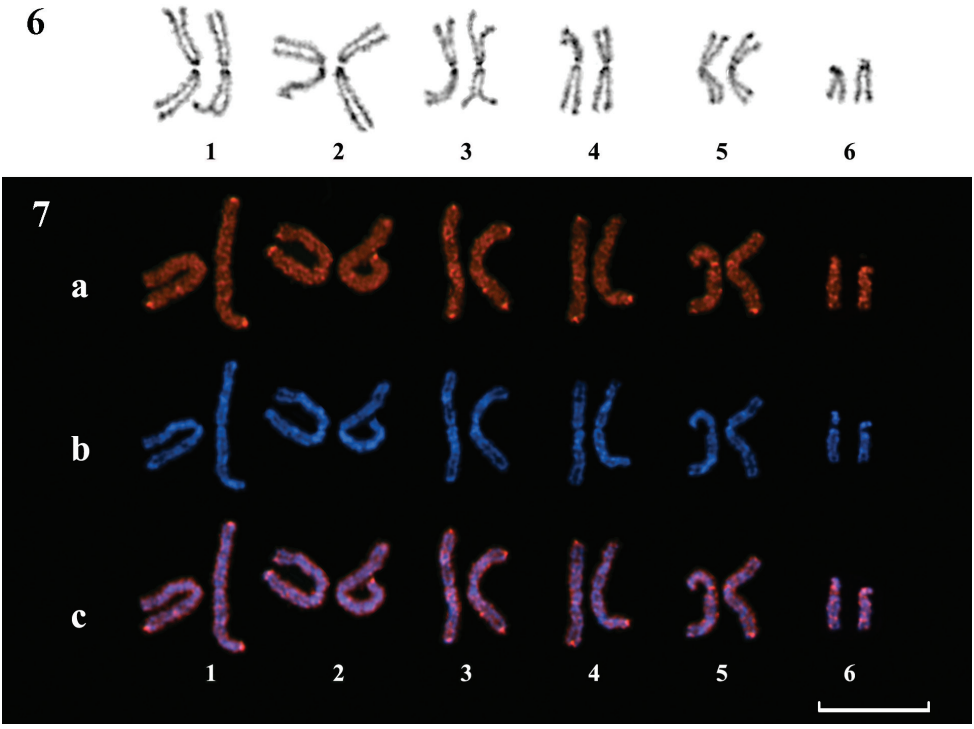


Figure 1. Female karyotypes of *Entedon cionobius* and *E. cioni*. Inverted DAPI staining. Bar = 10 μm.

CMA_3-positive bands on the long arms of the acrocentric chromosomes (Figs 2 and 3). CMA_3/MG staining also demonstrated analogous GC-rich bands in this region (Fig. 4). MG/DAPI staining revealed the same banding patterns as DAPI staining alone (data not shown).



Figures 2–5. 2 PI/DAPI-stained male metaphase plate of *E. cioni*. **a** PI staining **b** DAPI staining **c** superposition of **a** and **b 3** CMA_3/DAPI-stained female metaphase plate of *E. cionobius*. **a** CMA_3 staining **b** DAPI staining **c** superposition of **a** and **b 4** MG/CMA_3-stained female metaphase plate of *E. cionobius* **5** FISH with 45S rDNA probe on a female metaphase plate of *E. cionobius*. Green labels indicate hybridization signals. The chromosomes were counterstained with DAPI. Arrows on Figures 2-5 indicate DAPI-negative, PI- and CMA_3-positive NORs on acrocentric chromosome 6. Bar = 10 μm.



Figures 6–7. 6 Female karyotype of *E. cioni*. C-banding pattern and Giemsa staining **7** Female karyotype of *E. cioni*. Indirect immunodetection of 5-methylcytosine. **a** 5-methylcytosine distribution along chromosomes **b** DAPI counterstaining **c** superposition of **a** and **b**. Bar = 10 μm.

FISH using a 45S rDNA probe (Fig. 5) demonstrated distinct pericentromeric signals on the long arms of the acrocentric chromosomes. Thus, the 45S rDNA is located in the same position as the bright CMA_3-positive bands that were visualized after CMA_3/DAPI and CMA_3/MG staining.

The C-banding pattern (Fig. 6) was analogous to the fluorochrome banding pattern, although it differed in the intensity of staining at the centromeric and telomeric regions. Weakly stained smaller interstitial C-bands were also revealed.

5-methylcytosine immunodetection with fluorochrome-labeled antibodies revealed distinct positive signals in the telomeric regions of most chromosomes. Additionally, a few weaker centromeric and interstitial signals could also be seen. However, the nucleolus organizer region did not demonstrate visible positive signals (Fig. 7).

After DNA denaturation during FISH and 5-methylcytosine detection, DAPI counterstaining demonstrated a banding pattern that was rather similar to both the DAPI- and C-banding patterns (Figs 5, 7). Therefore, it was possible to identify most chromosomes after performing FISH or 5-methylcytosine immunodetection.

Discussion

The diploid karyotype of the previously studied *Entedon* species (Gokhman 2004) and the karyotypes observed in the species studied in the present paper are rather similar; they comprise five pairs of relatively large metacentric chromosomes and one pair of smaller acrocentric chromosomes. Moreover, this karyotype structure is also characteristic of the overwhelming majority of the family Eulophidae and is therefore considered a ground plan feature of the family Eulophidae (Gokhman 2002, 2004). The banding patterns revealed by DAPI staining on chromosomes of both of the studied species also appeared to be similar, therefore reliably confirming the high karyotypic similarity between the species. All these results are certainly not surprising since both *Entedon cionobius* and *E. cioni* belong to the same species group (see above).

Structural heterogeneity of chromosomes is characteristic of many animals, including insects (Rodionov 1999). Staining of mammalian chromosomes with DAPI, an AT-specific DNA-binding ligand, reveals banding patterns that are similar to the regular Q- and G-banding patterns. However, staining with GC-binding ligands revealed R-banding patterns that were inverse to the G-banding ones (Sumner 1994). G-like banding patterns were observed after the regular G-banding procedure of trypsin pretreatment and Giemsa staining in parasitic wasps that belong to the genus *Encarsia* Förster, 1878 (Aphelinidae) (Odierna et al. 1993, Baldanza et al. 1999). However, the nature of those banding patterns remained obscure. To study the characteristics of DAPI banding obtained during the present investigation, we performed chromosomal staining, both singly and in combination, with DNA-binding ligands of different specificity. We found that DAPI, an AT-binding ligand, CMA_3, a GC-binding ligand, and propidium iodide, a fluorochrome without preferential affinity for AT or GC pairs, produced the same banding patterns in all chromosomal regions, except for the NOR. Consecutive staining of human or other mammalian chromosomes with DAPI and AT-specific non-fluorescent chemicals, such as distamycin A or methyl green, was reported to produce specific banding patterns that are different from the regular DAPI patterns (Donlon and Magenis 1983, Schweizer and Ambros 1994). Although the molecular mechanisms of this staining are unclear, DA is generally considered to be more effective at displacing DAPI when the latter is bound to contiguous AT clusters instead of mixed AT/GC sequences (Burckhardt et al. 1993). Nevertheless, we found no difference between MG/DAPI and DAPI staining in the species studied. Thus, unlike the characteristic banding patterns of mammalian chromosomes, different fluorochromes produced similar banding patterns on the chromosomes of the two studied *Entedon* species. The patterns observed in the present study indicate differences in packing density between the various chromosomal regions rather than changes in AT/GC composition.

The rDNA sites are highly conserved in all eukaryotic organisms (Muravenko et al. 2001, Smit et al. 2007). Van Vugt et al. (2005, 2009) used a 45S rDNA probe originating from a wheat genome (Gerlach and Bedbrook 1979) to successfully visualize nucleolus organizers in the parasitic wasp *Trichogramma kaykai*. In the present work, FISH using the same 45S rDNA probe revealed the only DAPI-negative and PI- and

CMA_3-positive region in the studied species' chromosomes, and this region appears to be a NOR. This NOR is localized on the long arm of the sixth acrocentric chromosome, close to the centromere. The position of the NOR in the pericentromeric region of subtelocentric or acrocentric chromosome is typical for parasitic Hymenoptera of the superfamily Chalcidoidea. Nucleolus organizers have been localized on acrocentric chromosomes in a few species of the genus *Encarsia* (Aphelinidae) (Giorgini and Baldanza 2004), in the *Pnigalio soemius* (Walker, 1839) species complex and in *P. vidanoi* Navone, 1999 of the family Eulophidae (Bernardo et al. 2008, Gebiola et al. 2012). Moreover, localization of NORs on subtelocentric or acrocentric chromosomes was also detected in *Cotesia congregata* (Say, 1836) of the family Braconidae (Ichneumonoidea) (Belle et al. 2002). In all these cases, the nucleolus organizers were localized on the shorter arms of subtelocentric/acrocentric chromosomes. However, in *Melittobia australica* Girault, 1912 (Eulophidae), the nucleolus organizer was localized on the telomere of a large metacentric chromosome (Maffei et al. 2001). The karyotypes of the above-mentioned species bear a single NOR, whereas *Trichogramma kaykai* has two NORs with terminal localization on metacentric chromosomes 1 and 4 (Van Vugt et al. 2005, 2009). These results suggest that further studies are needed to adequately describe the real diversity in the number and localization of nucleolus organizers in parasitic Hymenoptera species.

Currently, 5-methylcytosine localization has been attempted only for mammalian and plant chromosomes (Miller et al. 1974, Schnedl et al. 1975, 1976, Bernardino et al. 2000, Ruffini Casteglione et al. 2002, Cremonini et al. 2003). However, DNA methylation is widespread in social Hymenoptera (Kronforst et al. 2008) where it can mediate nutritional control of caste determination (Kucharski et al. 2008). Nevertheless, the only successful attempt to investigate DNA methylation in insect chromosomes using specific antibodies was reported for the polytene chromosomes of certain Diptera, namely, *Drosophila* Fallen, 1823 and *Sciara* Meigen, 1803 (Eastman et al. 1980).

In many organisms where the distribution of 5-methylcytosine on chromosomes has been studied, high levels of DNA methylation in heterochromatic segments were detected, usually in telomeric and centromeric regions. The occurrence of intense DNA methylation in the analogous heterochromatic regions of *Entedon* chromosomes is therefore not surprising. However, highly functionally active chromosomal regions, such as NORs, are not intensively methylated in many organisms (Santoro and Grummt 2001), including the species here studied.

Conclusion

The present study supports previous hypotheses and provides new insights into the chromosomal structure of parasitic Hymenoptera. Specifically, several different DNA-binding ligands, such as propidium iodide, chromomycin A_3 and methyl green, were used for the first time for cytogenetic study of these insects. However, the similarity in banding patterns obtained through these ligands confirms that, unlike in mammals and some other groups, the bands represent differences in packing density along chro-

mosomes instead of differences in base composition. The karyotype structure of the *Entedon* species, and the family Eulophidae in general, appeared to be relatively conserved. However, we were able to demonstrate an unusual position for the nucleolus organizer in both examined species. For the first time in the order Hymenoptera, our data also visualize the presence of 5-methylcytosine in *Entedon* genomes in detectable amounts and its non-random distribution along the chromosomes. This allows the use of 5-methylcytosine immunodetection for the investigation of sex determination, cell differentiation and epigenetic regulation of Hymenoptera genomes.

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DATA PAPER

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Cytogenetic response of Scots pine (*Pinus sylvestris* Linnaeus, 1753) (Pinaceae) to heavy metals

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Abstract

We studied cytogenetic reactions of Scots pine seedlings to heavy metals – lead, cupric and zinc nitrates applied at concentrations 0.5 to 2000 μM. We determined the range of concentrations of heavy metals that causes mutagenic effect. Lead was found to cause the strongest genotoxicity as manifested by significant increase in the frequency of pathological mitosis, occurrence of fragmentations and agglutinations of chromosomes, various types of bridges, and a significant number of the micronuclei which were absent in the control. Possible cytogenetic mechanisms of the cytotoxic action of heavy metals are discussed.

Keywords

Scots pine, heavy metals, lead nitrate, zinc nitrate, cupric nitrate, mitosis, chromosomal abnormalities, micronuclei

Introduction

Over the last decade, heavy metals (HMs) have become the most dangerous and widely spread pollutants of the biosphere and especially soil (Titov et al. 2007). Increase in HMs content is a serious environmental problem. Mining, metallurgy, chemical industry, transport, and widespread use of mineral fertilizers and pesticides are among the major sources of HMs occurrence in the environment. Numerous studies have demonstrated that excessive concentrations of HMs accumulated in plants negatively

impact their growth, development and productivity. Apart from the toxic effect, HMs have a mutagenic (genotoxic) effect which is still not sufficiently studied (Steinkellner et al. 1998; Titov et al. 2007; Kulaeva and Tsyganov 2010).

The influence of HMs on plants (usually on grasses and more rarely on trees and shrubs) has been intensively studied by morphological, physiological, and biochemical methods (Bessonova 1991; Chakravarty and Srivastava 1992; Dovgalyuk et al. 2001; Seregin and Ivanov 2001; Fedorkov 2007). There are also data on the influence of HMs at the genetic and cytogenetic levels (Kim et al. 2003; Dobrzeniecka et al. 2011; Vandeligt et al. 2011). However, experimental studies on the cytogenetic responses of conifers to specific metals are not so numerous (Arduini et al. 1994; Prus-Głowacki et al. 2006; Yücel et al. 2008). Scots pine (*Pinus sylvestris* L.) is one of the most common forest forming species in Palaearctic. The investigation of cytogenetic responses of Scots pine seedlings to various HMs will help to estimate the genotoxicity and cytotoxicity of these metals as well as the metal resistance of the plants and develop criteria for selection of tolerant trees for reforestation in anthropogenically polluted areas.

Material and methods

The seeds of pine trees (*Pinus sylvestris* L.) from the Usmansky forest (the territory of the Voronezh State Biosphere Reserve; sector 80, site 22) were used. The conditions in this site are dry forest (A_1); the composition is 100% Scots pine. The Usmansky forest is a secondary forest, located along the watersheds of the Voronezh River and the Usman River, Voronezh province, Russia. The quantity of HMs in soil is not excessive (Protasova and Charykova 2011). According to cytogenetic analysis, this pine forest is considered as the benchmark of environmentally safe area (Butorina et al. 2007).

Seeds taken from 10 trees were mixed (in equal amounts from each tree) and placed in Petri dishes on moist filter paper and then germinated at room temperature. In the experimental variants the seeds were pre-soaked in the solutions of lead nitrate $Pb(NO_3)_2$, zinc nitrate hexahydrate $Zn(NO_3)_2 \cdot 6H_2O$, cupric nitrate trihydrate $Cu(NO_3)_2 \cdot 3H_2O$ and potassium nitrate KNO_3 of different concentrations (from 0.5 to 2000 μM) for 18 hours. Then the seeds were germinated in the same solutions in Petri dishes on moist filter paper at a room temperature for 5–7 days. The selected concentrations of HMs are stressful and correspond approximately to: $Pb(NO_3)_2$ – from 3.5 to 14 000 MAC (maximum allowable concentration), $Zn(NO_3)_2 \cdot 6H_2O$ – from 3.3 to 13 000 MAC, $Cu(NO_3)_2 \cdot 3H_2O$ – from 32 to 128 000 MAC (Dzhuvelikyan 1999). The seeds which were soaked and germinated in distillated water at the same exposure served as the controls. Potassium nitrate was used to exclude the side effect of the nitrate ion impact. The rootlets (reaching lengths of 5–15 mm) of the seedlings from the control and experimental samples were fixed at 9 am and 7 pm (at the peak of the mitotic activity in *Pinus sylvestris* L. in our conditions) in ethanol-acetic mixture (3 parts of 96% ethanol and 1 part of glacial acetic acid). The preparations for cytogenetic analysis stained in aceto-hematoxylin were made according to the technique described

earlier (Butorina and Kalaev 2000). More than 20 roots of seedlings (1 rootlet – 1 preparation) were studied for each sample (18 experimental ones and 1 control variant). At minimum 1000 cells were analyzed on each slide. The total number of analyzed cells was more than 380 000. Microphotographs were made with a DCM500 eyepiece digital camera (USB 2.0; WEBBERS MYscope 500 M).

The following parameters were revealed: 1) the mitotic activity of meristematic tissue (which was estimated by the mitotic index (MI)) calculated as a percentage of the number of dividing cells in prophase, meta-, ana-and telophase of mitosis to the total number of counted cells), 2) the percentage of cells in each mitosis phase to determine the duration of these phases, 3) the frequency and spectrum (types) of mitotic abnormalities (MPs) (the frequency was calculated as a ratio of the number of cells with abnormalities in the meta-, ana-, telophase of the mitosis to the total number of dividing cells viewed in %, spectrum of MPs represented as a percentage of each type of pathology to the total number of pathological mitoses), 4) the presence and frequency of cells with micronuclei was calculated as the percentage of cells with micronuclei to the total number of interphase cells counted, and 5) the proportion of cells with *n* number of nucleoli in interphase cells. The number of nucleoli was counted in 500–600 interphase cells for each preparation.

Statistical processing of the data was performed with the help of the statistical program package Stadia and Statistica. The procedure of grouping the data and their treatment are described in the work of Kulaichev (2006). The experimental samples were compared according to the mitotic activity and nucleolar characteristics using Student's t-test. The comparison of samples in terms of their MPs was carried out using van der Waerden rank X-test, because this feature does not follow the normal distribution. The normality check of distribution was performed using the chi-square test. The influence of the "type of metal" factor was determined using Kruskall-Wallis nonparametric univariate analysis of variance test.

Results and discussion

Variation of cytogenetic parameters in the root meristem of Scots pine seedlings from the control and experimental samples is presented in Table 1.

Mitotic activity. Mitotic activity determines growth energy. Since the mitotic index (MI) is a rather stable parameter at a given time, its change may reflect a mutagenic influence of environmental factors on studied objects (Butorina and Kalaev 2000).

The mitotic index decreased significantly in the seedlings of experimental samples compared with the control under exposure to all three HM salts (lead, zinc and cupric nitrate) (Table 1). However, the decrease did not exceed 1.5% and the MI was in the narrow corridor of 6.0–6.7% regardless of the concentration of HMs. When the experimental samples were compared, the differences were observed only at extreme concentrations. We suppose that a slight decrease in the MI was caused by an inhibition of the existing dividing cells at different stages of mitosis as a response to HMs.

Table 1. Average cytogenetic parameters of the root meristem of Scots pine resulting from various exposure concentrations of heavy metals (lead, zinc and cupric nitrates) and potassium nitrate. Differences from the control significant at: ** $P < 0.01$, *** $P < 0.001$.

Cytogenetic parameter,%	Control	0.5 μM	5 μM	50 μM	500 μM	1000 μM	2000 μM
Lead nitrate							
MI	7.5±01	7.4±0,1	6.6±0.1***	6.4±0.1***	6.1±0.1***	6.1±0.1***	6.0±0.2***
MPs	0.4±0.2	0.5±0.2	4.4±0.4***	5.8±0.3***	7.4±0.3***	8.2±0.4***	11.1±0.4***
micronuclei	0	0.01±0.005	0.05±0.012	0.13±0.014	0.21±0.014	0.31±0.015	0.43±0.025
Zinc nitrate							
MI	7.5±0.1	7.4±0.1	6.8±0.1**	6.7±0.1***	6.6±0.1***	6.6±0.1***	6.4±0.2***
MPs	0.4±0.2	0.4±0.2	3.2±0.4***	5.1±0.5***	5.5±0.4***	7.1±0.4***	9.5±0.5***
micronuclei	0	0	0.02±0.005	0.05±0.006	0.11±0.012	0.19±0.013	0.23±0.015
Cupric nitrate							
MI	7.5±0.1	7.3±0.1	6.7±0.1***	6.6±0.1***	6.6±0.1***	6.5±0.1***	6.1±0.2***
MPs	0.4±0.2	0.5±0.2	2.3±0.4***	3.7±0.4***	4.7±0.4***	5.9±0.4***	6.3±0.5***
micronuclei	0	0	0.01±0.006	0.04±0.014	0.11±0.014	0.18±0.016	0.25±0.016
Potassium nitrate							
MI	7.5±0.1	7.4±0.1	6.4±0.1***	6.1±0.1***	6.1±0.1***	6.2±0.1***	6.6±0.2**
MPs	0.4±0.2	0.3±0.1	0.4±0.2	0.4±0.2	0.5±0.3	0.3±0.1	0.4±0.2
micronuclei	0	0	0	0	0.02±0.009	0.01±0.006	0.04±0.014

A comparison of the frequency of cells at different stages of mitosis revealed that under conditions of stress (experimental samples) there was an increase in the proportion of cells at metaphase, and especially the metaphase-anaphase transition (meta-anaphase) (Fig. 1), as illustrated by the influence of lead nitrate (Table 2). The inhibition of mitosis manifested in a significant (8.5 times for Pb; 7.8 times for Zn; 11.6 times for Cu) increase in the proportion of cells at the intermediate stage of meta-anaphase could be a result of blocking microtubule polymerization as was noted by different authors (Rieder and Palazzo 1992; Wierzbicka 1994; Seoane and Dulout 2001; Thier et al. 2005). For example, it was shown that lead reacts with 2 out of the 18 thiol groups in tubulin dimers of non-polymerized microtubules as well as in already formed ones (Faulstich et al. 1984; Klapheck et al. 1995).

The change of the duration of cells' passage through the stages of mitosis under the influence of HMs may be associated with the checkpoint activation. In cell cycle, transitions from G_1 cells to S-phase from G_2 to mitosis and metaphase to anaphase are viewed as critical stages in which a temporary stop of the cell cycle and check of the genetic material's integrity take place. Therefore, the "control point" of the cell cycle is a mechanism that protects the dividing cells against lethal mitosis by activation of repair systems for DNA damage or self-destruction of heavily damaged cells via apoptosis. Thus, it is possible to consider the change in duration of cells' passage in mitosis (an increase in the number of cells in metaphase and meta-anaphase) as one of the mechanisms of adaptation to stressors and maintenance of homeostasis in cell populations of Scots pine seedlings under the influence of Pb, Zn and Cu.

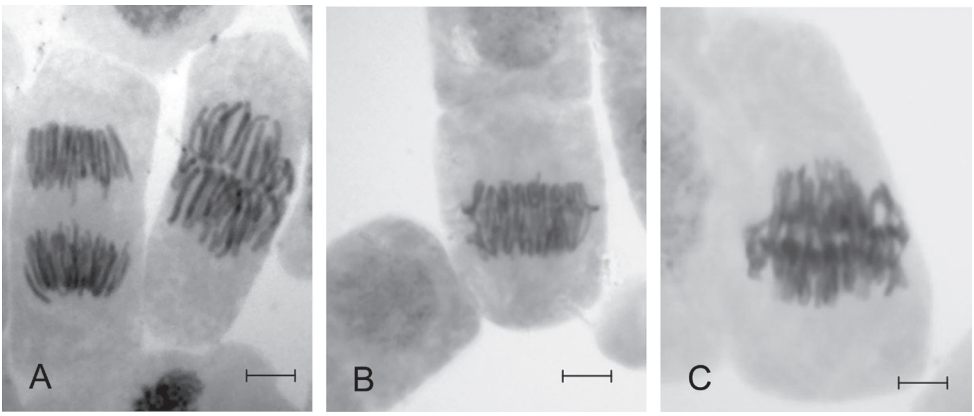

Figure 1. Cells of the root meristem of Scots pine at metaphase and anaphase of mitosis **A** and at the transitional stage of meta-anaphase **B** and **C**. Bar = 10 μm.

Table 2. A proportion of cells in % at each stage of mitosis in the root meristem of Scots pine subjected to Pb nitrate. Significance (from control): ** $P < 0.01$, *** $P < 0.001$.

Variant	Mitosis stage				
	Prophase	**Metaphase**	**Meta-anaphase**	**Anaphase**	**Telophase**
Control	10.4±0.5	30.2±0.9	1.2±0.2	28.4±0.5	29.8±0.4
0.5 μM	9.8±0.4	31.2±1.2	1.3±0.2	27.6±0.7	30.1±0.4
5 μM	10.2±0.5	35.2±1.3***	5.3±0.3***	22.3±1.3***	27.0±1.3**
50 μM	6.6±0.3***	35.9±0.9***	6.8±0.3***	25.6±0.7***	25.1±0.7***
500 μM	9.9±0.6	34.4±0.9***	8.8±0.4***	21.3±0.8***	25.6±0.8***
1000 μM	12.0±0.7***	32.1±1.1**	9.1±0.2***	22.3±1.2***	24.5±0.7***
2000 μM	13.8±0.9***	31.0±1.0	10.2±0.4***	20.9±1.1***	24.1±0.8***

The obtained data on the impact of equal concentrations of various HMs salts on the mitotic activity of root meristem cells allow us to estimate their cytotoxicity. For example, although the decrease in the MI was similar (Table 1) for all HMs salts, the numbers of cells in meta-anaphase were significantly different (Fig. 2). The most toxic under our experimental conditions was Cu; it was followed by Pb and Zn, in agreement with published data (Fargašová 1998; Montvydienė and Marčiulionienė 2004). Thus, the cytotoxic response of Scots pine seedlings to HMs can be presented from the strongest to the weakest as follows: Cu > Pb > Zn.

Mitotic pathologies and the micronucleus test. It has been shown for Scots pine that the level and spectrum of mitotic pathologies (MPs), the frequency of cells with micronuclei and the number of nucleoli in the cell are the most sensitive cytogenetic parameters to the anthropogenic pollution, objectively reflecting the state of their genetic system (Butorina and Kalaev 2000).

A significant increase of MPs was found for all tested salts of HMs (lead, zinc and cupric nitrates) starting from the concentration of 5 μM (selected as a threshold).

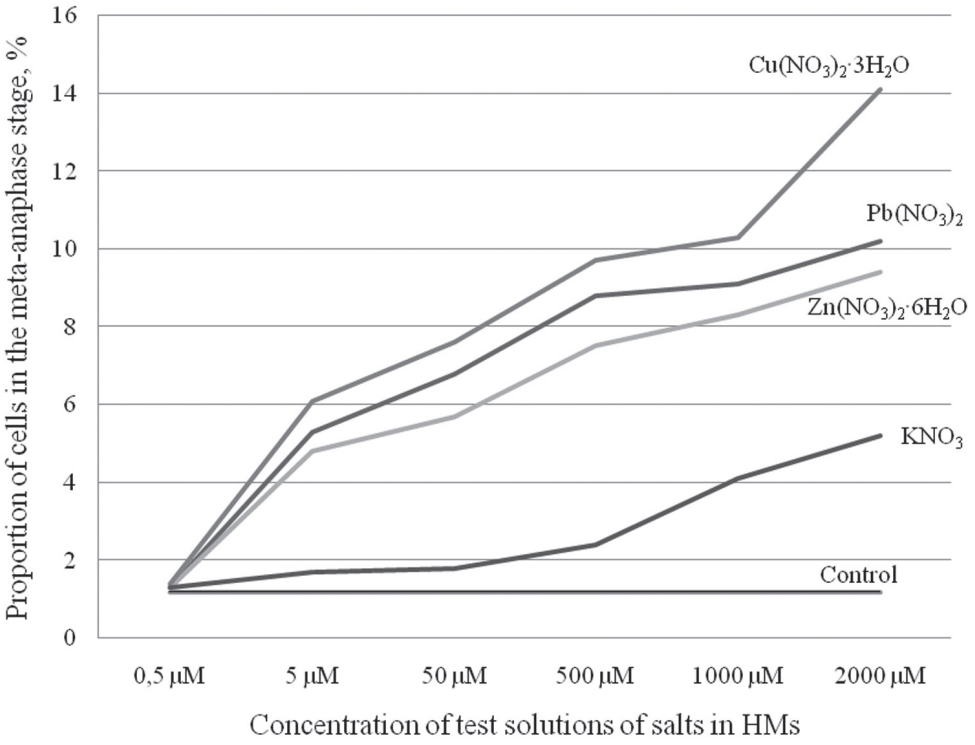

Figure 2. Average percentage of cells at the meta-anaphase stage by concentration of selected salts (lead, zinc, cupric and potassium nitrates) in comparison with the control.

This concentration corresponds to 35 MAC (maximum allowable concentration) for Pb, 33 MAC for Zn and 320 MAC for Cu. The usage of so high concentration in our experiment is explained by the short duration of HMs infulence. Typically, the value of MAC on HMs-polluted territories is lower in natural conditions, but it can come up to 35 MAC for Pb (Dzhuvelikyan 1999). In anthropogenic conditions plants are chronically influenced by HMs so the accumulation of pollutant in the plant tissue should be considered. Our research on the reaction of Scots pine experiencing a chronic combined effect of the influence of pollutants including HMs in the district of the Novolipetsk metallurgic factory revealed substantial changes of cytogenetic parameters compared to those of controls on ecologically favorable territory (Mashkina et al. 2009). There are data (Dobrzeniecka et al. 2011) that genetic variation of *Picea mariana* (Miller, 1768) defined with ISSR markers depends on the degree of moistening of the soil containing the HMs group. The genetic variation was higher in the population of *Picea mariana* growing on the wet lands as compared to those growing on the dry land. But cytological analysis of black spruce seeds from metal-contaminated and uncontaminated areas showed normal mitotic behavior during prophase, metaphase, anaphase, and telophase. There is no research on the influence of the specific HMs on the mitosis under field conditions.

With increase in HMs concentration the frequency of the MPs increased. Starting with the concentration 50 μM for Pb and Zn, the average value of the MPs exceeded normal values of spontaneous mutation level in central Russia by up to 5% (Butorina et al. 2007). Moreover, the sensitivity of the parameter "MPs frequency" was significantly higher than that of MI. The changes (comparing to control) were 11–27 fold (depending on the concentration) for Pb, 8–23 fold for Zn and 5–15 fold for Cu, whereas the MI changes were minimal, and the cell proportion at the stage of meta-anaphase were 11 times greater (maximum for Cu) (Table 1).

The spectrum of MPs in Scots pine seedlings of experimental samples was manifested by the same abnormalities as identified in control (chromosome segregation and lagging in anaphase, single bridges in ana-telophase) as well as by the new ones such as chromosome fragments in prophase, metaphase, anaphase, chromosome agglutination; chromosome segregation in metaphase and anaphase; different types of bridges (single or multiple, broken bridges, bridge and chromosome fragments, bridge and chromosome lagging etc.) (Fig. 3). The predominance of the lagged chromosomes and marginalized groups of chromosomes observed in the whole spectrum (up to 51.6% for lead) could be due to the inhibited spindle caused by HMs ions. It could also be one of the reasons for a significant number of micronuclei. Similar abnormalities in the control were apparently repaired as micronuclei were not identified. The emergence of chromosome fragments in experimental samples and various types of complex bridges, which totaled up to 48.4% (for Pb), indicate increased levels of mutation (chromosomal rearrangements) under influence of HMs.

The tested HMs generally caused similar cytogenetic response. Nevertheless, we noticed some specificity of the induced abnormalities. For example, Pb to a greater extent (from 0.4 to 19.8%) caused an appearance of chromosome fragments in prophase and metaphase. Zn had the widest range of induced MPs, including chromosome fragmentation (from 0.4 to 7.3%) and agglutination (0 to 3.6%) and multiple bridges. Cu mainly caused agglutination (0 to 8.8%) and less frequently chromosome fragmentation (from 0 to 3.7%) (Fig. 4).

The differences between the experimental variations were particularly clearly observed in the frequency of occurrence of micronuclei in interphase cells (Table 1). Micronuclei were not identified in the controls. The maximum quantity of micronuclei was observed for Pb (from 0.01 to 0.43% depending on the concentration used), whereas it was lower and almost identical for Zn and Cu (0-0.23% for Zn and 0-0.25% for Cu). The presence of micronuclei indicates unrepaired damage of chromosomal material. It leads to cytogenetic instability in cell populations (Ilyinskikh et al. 1998, Mashkina et al. 2009). Thus, the inhibitory effect of HMs was extended to DNA repair enzymes, as proved by a significant increase in the number of cells containing DNA fragments, bridges, and micronuclei.

The seedlings treated with potassium nitrate showed no significant differences in MP frequency compared with the control (Table 1). Micronuclei were detected only at extreme concentrations (500, 1000 and 2000 μM) and their number was negligible (0.02-0.04%), which excludes the impact of nitrate-ion present in all three salts of HMs (Pb, Zn and Cu).

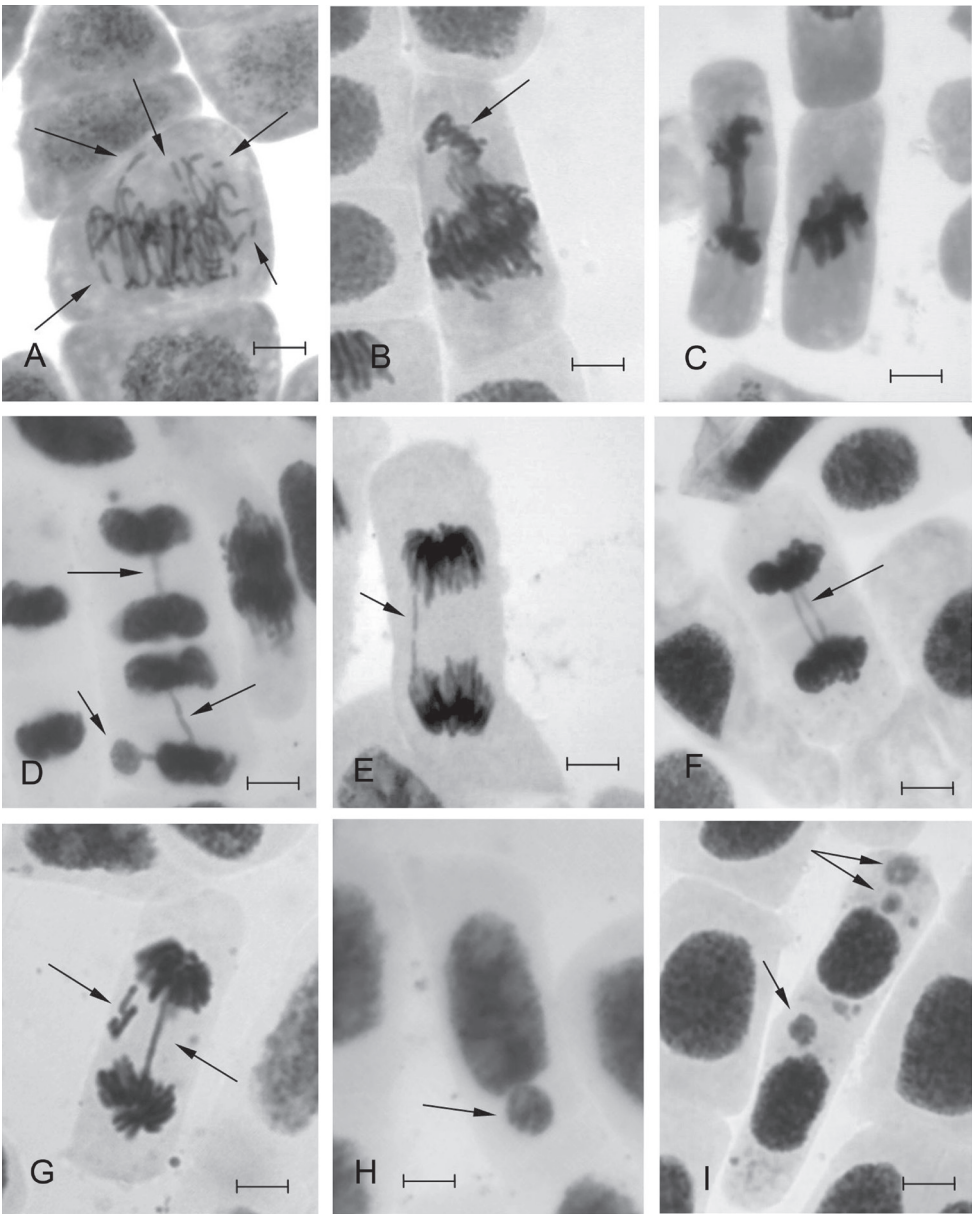

Figure 3. Types of mitotic pathologies and micronuclei in interphase cells found in the root meristem of Scots pine seedlings under exposure to HMs: **A** chromosome fragmentation in metaphase **B** chromosome isolation at meta-anaphase **C** multiple bridge and chromosome agglutination **D** bridge and micronucleus **E** broken bridge **F** double bridge **G** bridge and lagging chromosome fragments **H, I** micronuclei. Bar = 10 μm.

According to the results Kruskall-Wallis nonparametric univariate analysis of variance test, lead had the greatest impact on the frequency of MPs (Fig. 4A) and the number of cells with micronuclei (Fig. 4B). The impact of Zn and Cu was not so great.

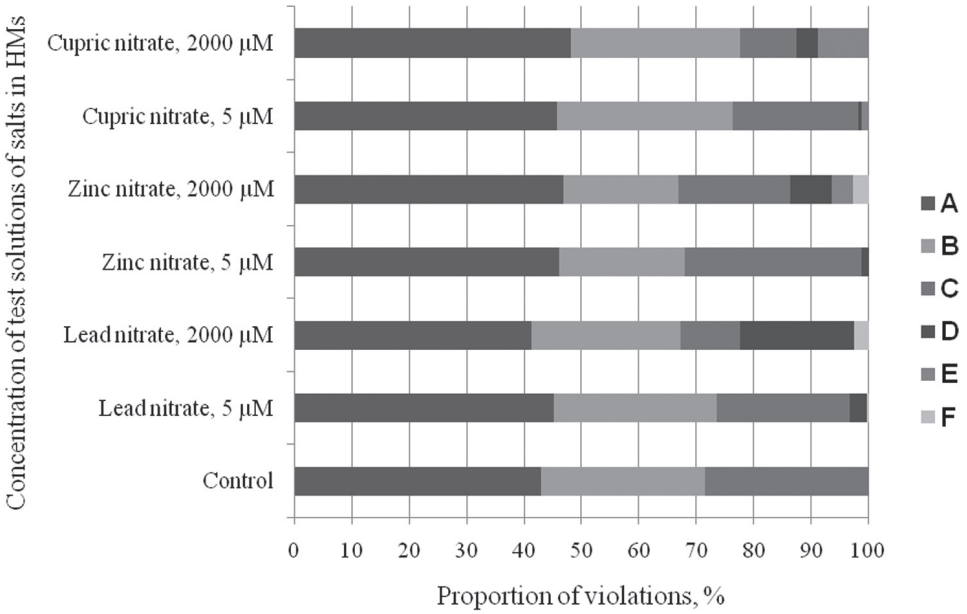

Figure 4. MPs spectrum in cells of the root meristem of Scots pine treated with salts of different concentration: **A** chromosome segregation in metaphase and anaphase **B** bridges **C** lagged chromosomes in anaphase **D** chromosome fragmentation **E** chromosome agglutination **F** multiple violations.

The observed differences were significant at $P < 0.001$. Thus, according to the data on the frequency of micronuclei and MPs, the mutagenic response of Scots pine seedlings to HMs can be presented from the strongest to the weakest as follows: Pb > Zn > Cu.

It was shown (Evseeva et al. 2005) that the toxic and mutagenic action of HMs is based on their ability to bind with amino acids and proteins. Metal ions in enzymes can be also substituted by HMs. HMs induce the formation of reactive oxygen species, protein denaturation, leading to the disruption of cellular metabolism, the processes that cause single breaks of DNA, which is a signal to a change in gene expression. Moreover, the mutagenic effect of HMs detected by the frequency of chromosome aberrations appears at lower concentrations than their cytotoxic effect.

It is well known that Cu and Zn are trace elements essential for cell metabolism, but their excess of the normal physiological level results to a negative impact. Several mechanisms of HM detoxification (including Cu and Zn) that ensure the maintenance of cellular homeostasis and metal resistance increase in plants have been described in a number of papers. These include an immobilization of the cell wall (Zornoza et al. 2002), formation of complexes with chelators (Sanjaya et al. 2008), synthesis of stress proteins (Clemens 2001), synthesis of metallothioneins and phytochelatins (Zhigang et al. 2006; DalCorso et al. 2008; Prévéral et al. 2009). Non-specific reactions to the action of HMs include increased activity of antioxidant enzymes (including peroxidase) that neutralize free radicals and peroxides formed as a result of oxidative stress, rendering the damaging effect on cells (Kuznetsov 2006). Pb is not necessary for liv-

ing organisms and its cellular metabolism is still poorly studied. Apparently, Pb can undergo partial detoxification as described above because of some similarity (to Ca, for example) of its physical and chemical properties (mainly valency).

The experiments with Scots pine showed (Belousov and Zemlyanukhina 2011) that cytogenetic parameters (the meta-anaphases proportion, the MPs level and spectrum, the micronuclei frequency) were more sensitive to Pb than the activity of a number of respiratory enzymes (including peroxidase – a marker of stress and adaptation), since the changes in the activity of the latter occurred at higher concentrations (500 μM) in contrast to the cytogenetic effects (starting at 5 μM).

Nucleolar activity. The role of the nucleolus in metabolic processes is due to its involvement in the biosynthesis of cell rRNA which is necessary for protein biosynthesis. It is known that nucleolar activity in pines can vary within wide limits manifested by the varying number of nucleoli (1 to 12 nucleoli in one nucleus). The number of nucleoli increases under extreme conditions (Muratova 1999, Haydarova and Kalashnik 1999, Butorina and Kalaev 2000). Cells with 3–5 nucleoli (mostly cells with 4 nucleoli) dominated in the control. It is normal for Scots pine (Butorina et al. 2007) and it indicates the predominant activity of nucleolar organizers of two or three pairs of chromosomes. In all experimental samples treated with HMs cells with 5–8 nucleoli (with maximum number of cells containing 6 nucleoli) prevailed. Moreover, the number of cells with the maximum number of nucleoli (10–12 nucleoli in the nucleus) significantly increased compared with the controls (3–5 folds increase, on average). This may be an indicator of metabolic activity enhancement (activation of rRNA genes, ribosomes and protein synthesis) under the influence of HMs as a regulatory mechanism to facilitate the increased protein metabolism in seedlings with MPs increased frequency.

Conclusion

The presented data show the high genotoxic effect of zinc, cupric and, especially, lead nitrates on Scots pine seedlings. With an increase in HMs concentration (from 5 μM), there were significant changes in cytogenetic parameters. 1) There was a marked inhibition of mitosis manifested in a significant increase in the number of cells in metaphase and intermediate meta-anaphase, which may be caused by blocking the polymerization of tubulin spindle microtubules. However, a decrease in mitotic activity may provide additional time to repair damage (small defects) of chromosomal material in the transition of cells through the "checkpoint" cell cycle. 2) The rate (up to 5–27 fold depending on the concentration of HMs) increased and the spectrum of MPs expanded. Specific chromosome damage, not observed in controls appeared, such as a large number of chromosome fragments (up to 19.8%) and agglutination (8.8%) and a significant amount of micronuclei in interphase cells (up to 0.43%) which indicated the inhibiting impact of HMs on the enzymes of DNA repair systems. 3) The cytogenetic adaptive reactions of Scots pine to the

action of HMs included increased cell metabolism (activation of ribosomal rRNA genes, as well as the synthesis of additional, likely stress proteins) due to an increase in cells with the maximum number of nucleoli (10–12) in the nucleus. 4) The data on MPs level and the micronuclei frequency indicate the strength of genotoxic (mutagenic) impact of HMs affecting Scots pine seedlings germination as Pb > Zn > Cu. We show that the most sensitive and suitable for assessing HMs genotoxicity in the early stages of development are the PM level and spectrum, the number of cells with micronuclei and the nucleolar activity.

Acknowledgments

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