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Comparative cytogenetics of Neotropical cichlid fishes (*Nannacara*, *Ivanacara* and *Cleithracara*) indicates evolutionary reduction of diploid chromosome numbers

Lucie Hodaňová[1], Lukáš Kalous[1], Zuzana Musilová[1,2,3]

1 *Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic* **2** *Laboratory of Fish Genetics, Institute of Animal Physiology and Genetics AV CR, Libechov, Czech Republic* **3** *Zoological Institute, University of Basel, Switzerland*

Corresponding author: *Zuzana Musilová* (zuzmus@gmail.com)

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Abstract

A comparative cytogenetic analysis was carried out in five species of a monophyletic clade of neotropical Cichlasomatine cichlids, namely *Cleithracara maronii* Steindachner, 1881, *Ivanacara adoketa* (Kullander & Prada-Pedreros, 1993), *Nannacara anomala* Regan, 1905, *N. aureocephalus* Allgayer, 1983 and *N. taenia* Regan, 1912. Karyotypes and other chromosomal characteristics were revealed by CDD banding and mapped onto the phylogenetic hypothesis based on molecular analyses of four genes, namely cyt *b*, 16S rRNA, S7 and RAG1. The diploid numbers of chromosomes ranged from 44 to 50, karyotypes were composed predominantly of monoarmed chromosomes and one to three pairs of CMA_3 signal were observed. The results showed evolutionary reduction in this monophyletic clade and the cytogenetic mechanisms (fissions/fusions) were hypothesized and discussed.

Keywords

Cichlid cytotaxonomy, cyt b, 16S rRNA, S7-1, RAG1 phylogeny, karyotype differentiation, CMA_3 phenotypes, Cichlasomatini

Introduction

Cichlids are a species-rich group of ray-finned fishes (Actinopterygii), distributed in tropical and subtropical freshwaters of Africa and South and Central America, Texas, Madagascar, the Middle East, India and Sri Lanka (Kullander 1998). As a third largest fish family (Eschmeyer and Fricke 2012) cichlids represent highly evolutionarily successful fish lineage and it is considered that no other family of vertebrates exceeds cichlids in a number of varieties, shapes, colors and especially in ecological and trophic specializations (Kocher 2004).

In general, genomes of ray-finned fishes are known for high evolutionary dynamics among vertebrates, which is reflected in huge genome-architecture variability (Mank and Avise 2006). The diploid chromosome number (2n) studied in 615 Actinopterygian species ranges from 22 to 250, but over a half of the species possess the conservative number of 2n = 48 – 50 chromosomes (29.3% have 2n = 48 and 25.4% have 2n = 50; Mank and Avise 2006). The most frequent fish karyotype, i.e. 2n = 48 (n=24), is also recognized as an ancestral karyotype of the whole Teleostei (Ohno et al. 1969, Nakatani et al. 2007).

In total, over 190 cichlid species have been cytogenetically analyzed and the karyotype formula was determined for 157 of them (Arai 2011). Available cytogenetic data in cichlids show that the diploid chromosome numbers range from 2n=32 to 2n=60, but more than 60% of the examined species show the ancestral karyotype with 2n=48, which mostly dominates in the Neotropical cichlid lineage (Feldberg et al. 2003).

In the past only few species were analyzed and Neotropical cichlids were considered a karyotypically conservative group due to the frequent findings of 48 chromosomes (Thompson 1979, Kornfield 1984). Later, Marescalchi (2004) and Poletto et al. (2010) demonstrated much higher variability in the chromosome number and hypothesized that the ancestral karyotype of the Neotropical cichlids underwent significant changes in structure in several lineages, which led to extensive karyotype diversification. Further, many species possess the similar 2n=48, but differ in karyotype structures, which brings additional evidence of the karyotype differentiation due to the intra-chromosomal rearrangements like centromeric shifts (Feldberg et al. 2003). It is likely that at least some different lineages coincidentally converged to the same number of chromosomes from different ancestral stages but the mechanisms of why there is certain favorable number of chromosomes remains still unknown (Mank and Avise 2006).

Dwarf cichlids of the genus *Nannacara* Regan, 1905, and its relatives, genera *Ivanacara* Römer & Hahn, 2007 and *Cleithracara* Kullander & Nijssen, 1989 represent a well-defined evolutionary lineage of acaras (NIC-clade of the tribe Cichlasomatini, Musilová et al. 2008) distributed mostly in rivers of the Guyana shield, as well as in the Rio Negro basin, and the Amazon and Orinoco deltas. This group includes seven known species, four in the genus *Nannacara*, then two species recently extracted from *Nannacara* to the genus *Ivanacara* (Römer and Hahn 2007), and the monotypic genus *Cleithracara*, which is basal to all the others. The cytogenetics of this clade remains poorly known since only two species of this group, *Cleithracara maronii* (Steindach-

ner, 1881) with 2n=50 (Marescalchi 2004) and *Nannacara anomala* Regan, 1905 with 2n=44 (Thompson 1979) have been previously investigated.

In this study we present karyotypes and other chromosomal characteristics as revealed by CDD banding in five species of monophyletic clade of neotropical Cichlasomatine cichlids, namely *Cleithracara maronii*, *Ivanacara adoketa* (Kullander & Prada-Pedreros, 1993), *Nannacara anomala*, *Nannacara aureocephalus* Allgayer, 1983 and *Nannacara taenia* Regan, 1912. We further mapped the results onto the phylogenetic hypothesis from molecular analyses based on four genes. We discuss possible scenario of the karyotype evolution of the clade of dwarf cichlids within the tribe Cichlasomatini.

Materials and methods

Materials

The species included in the present study are listed in Table 1. Most of the individuals originated from aquarium trade from different breeders. Further, various collectors or ornamental-fish importers donated several samples for DNA analysis. Species were identified following Kullander and Nijssen (1989), Kullander and Prada-Pedreros (1993) and Staeck and Schindler (2004), and part of the analyzed fish was deposited in ICCU (Ichthyological Collection of Charles University, Prague). See Table 1 and Table 2.

Cytogenetic analyses

Chromosomes were obtained by non-destructive isolation procedure from caudal fin regenerates as developed by Völker et al. (2006) and modified by Kalous et al. (2010). This method is based on regeneration of the caudal fin tissue after cutting a small part (2–3mm) from its margin. After five to seven days the regenerated tissue was cut and incubated in the solution with colchicine for two hours at room temperature. A few drops of fixative (methanol, acetic acid 3:1) were added to the tissue after this incubation and it was placed for 30min at 4°C. The tissue was washed twice in fixative, always staying for 30min at 4°C after the wash. Next, the tissue was placed into a drop of 20% acetic acid and gently mashed through a fine sieve. The suspension was dropped on a slide on a hot plate (45°C). After 20 seconds the drop of suspension was sucked up from the slide and dropped to a different place in the slide. Metaphase chromosomes were stained in 4% Giemsa solution in phosphate buffer (pH=7). Generally 5–50 metaphases per individual were evaluated. Chromosomes were classified according to Levan et al. (1964), to be consistent with most of the recent studies on cichlid fishes (Marescalchi 2004, Fedlberg et al. 2003, Poletto et al. 2010) and arranged to karyotypes by using ADOBE PHOTOSHOP, version CS7. The CDD fluorescent banding (Chromomycin A_3/methyl green/DAPI) was performed following Mayr et al. (1985) and Sola et al. (1992).

Table 1. Sample list for the present study. Details on individuals of cichlids investigated for the molecular genetics. Outgroup data were used from the original study (Musilová et al. 2008, 2009).

Individuals used in molecular phylogenetic analyses:			Accesion numbers in GenBank				Sample voucher
species	sample code	origin	cytb	16SrRNA	S7	RAG1	
Geophagus brasiliensis	*outgroup - used from GenBank*		EF470895	EU888080	EU199082	EU706360	-
Bujurquina vittata	*outgroup - used from GenBank*		EF432951	EF432892	EF432984	EU706385	-
Aequidens metae	*outgroup - used from GenBank*		EF432927	EF432882	EF432974	-	-
Laetacara thayeri	*outgroup - used from GenBank*		AY050608	EF432909	EF433001	EU706401	-
C. maronii	Cleith	aquarium trade	AY050614	EF432901	EF432993	EU706394	ICCU 0736
N. (I.) adoketa	ADO	aquarium trade	EF432946	EF432903	EF432995	EU706396	ICCU 0745
N. (I.) adoketa	In06	Rio Inirida	KJ136667	-	KJ136659	-	ICCU 1001
N. (I.) adoketa	In03	Rio Inirida	KJ136668	-	KJ136660	-	ICCU 1002
N. anomala	ANO	aquarium trade	AY050618	EF432898	EF432990	EU706391	ICCU 0746
N. anomala	NaD	Orinoco delta	KJ136669	KJ136671	KJ136661	-	ICCU 1004
N. anomala **"Suriname"**	WSN	F1 progeny	-	-	KJ136654	-	-
N. aureocephalus **"blue"**	RNA01	aquarium trade	-	KJ136673	KJ136663	-	ICCU 1005
N. aureocephalus **"blue"**	RNA03	aquarium trade	-	KJ136674	KJ136664	-	-
N. aureocephalus **"blue"**	RNA04	aquarium trade	-	KJ136675	KJ136665	-	-
N. aureocephalus	AUR	aquarium trade	EF432939	EF432899	EF432991	EU706392	ICCU 0747
Nannacara **sp.**	SAR	import/unknown	-	KJ136670	KJ136655	KJ136666	ICCU 1003
N. prope aureocephalus **"brown"**	AurBrown01	aquarium trade	-	KJ136672	KJ136662	-	-
Nannacara sp. **"Soumourou"**	NSP01	F1 progeny	-	-	KJ136656	-	-
Nannacara sp. **"Oyapock"**	NSP02	F1 progeny	-	-	KJ136657	-	-
Nannacara sp. **"Oyapock"**	NSP03	F1 progeny	-	-	KJ136658	-	-
Nannacara sp.	AF045860	GenBank	-	AF045860	-	-	-
N. taenia	TAE	aquarium trade	EF432921	EF432900	EF432921	EU706393	ICCU 0749

Table 2. Sample list for karyotypes analysis.

Individuals used in cytogenetic analyses (all from aquarium trade):		
Species	**Number of analyzed individuals**	**Sex**
C. maronii	3	undifferentiated
I. adoketa	3	2× male, 1× female
N. anomala	5	3× male, 2× female
N. aureocephalus	3	undiferentiated
N. taenia	3	undiferentiated

Molecular genetic analyses

DNA was extracted from the ethanol-preserved samples by the commercially available kits (QiaGen), and four target genes (cyt b, 16S rRNA, S7 first intron, RAG1) were amplified by PCR using primers according to Musilová et al. (2009). Sequences of the PCR products were obtained by commercial sequence-service company (Macrogen, South Korea, Netherlands). Sequences were aligned in BIO EDIT (Hall 1999) software and genes were concatenated for the bayesian analysis in MRBAYES 3.2. (Ronquist et al. 2012). Analysis parameters were: number of generations = 10,000,000, number of chains = 4, number of runs = 2, model set for every gene separately (and unlinked) based on the jModeltest (Posada 2008) results. Three additional species (*Bujurquina vittata, Aequidens metae* and *Laetacara thayeri*) from the same taxonomic tribus Cichlasomatini as *Nannacara* + *Ivanacara* were analyzed as well, and one species of the different tribus Geophagini (*Geophagus brasiliensis*) was determined as an outgroup for the phylogenetic analysis. Sequences were uploaded to GenBank (Table 1).

Results

Karyotype characteristics

Results are summarized in Fig. 1 and Table 3. Examined individuals of the species of genera *Nannacara, Ivanacara* and *Cleithracara* showed the diploid chromosome number 2n = 44 to 50 chromosomes. All three species of the genus *Nannacara* possessed 44 chromosomes and karyotype composed of 18 metacentric (m)-submetacentric (sm)+26 subtelocentric (st)-acrocentric (a) or 16m-sm+28st-a chromosomes, while *Ivanacara adoketa* had 2n = 48 and karyotype of 16m-sm+32st-a chromosomes, and *Cleihtracara maronii* had 2n = 50 composed of 14sm+36st-a chromosomes. Karyotypes of all studied species are shown in Fig. 1.

CDD fluorescence

In the karyotypes of four studied species, namely *C. maronii*, *I. adoketa*, *N. anomala*, and *N. taenia*, the CMA_3-positive signals were found on one chromosome pair,

Table 3. Karyotype characteristics of the South American dwarf cichlids, including the diploid number of chromosomes (2n), chromosome categories, and CMA_3 phenotype.

Species	*2n*	Karyotype	*CMA_3* signals
Cleithracara maronii	50	14sm+36st-a	1 sm pair
Ivanacara adoketa	48	16m-sm+32st-a	1 st-a pair
Nannacara anomala	44	18m-sm+26st-a	1 m-sm pair
Nannacara aureocephalus	44	18m-sm+26st-a	3 m-sm pair
Nannacara taenia	44	16m-sm+28st-a	1 st-a pair

although probably not homologous in different species. In *C. maronii* the CMA_3-positive signals were located on terminal parts of the largest m-sm chromosome pair, whereas in *I. adoketa* and *N. taenia* the CMA_3 signals were located a chromosome pair from st-a group, terminal parts in *N. taenia* and around the centromere in *I. adoketa*. In *N. anomala* the CMA_3 signals were found on the terminal parts of a chromosome pair from m-sm group, but not on the largest pair. Contrarily, in the karyotype of *N. aureocephalus*, the CMA_3 signals were located on three m-sm chromosome pairs including the largest chromosome pair in the centromeric region. See Table 3 for more detail about the karyotype formulas and CMA_3 phenotypes and Fig. 1 for representative metaphases and results of different staining steps.

Phylogenetic analysis and karyotype differentiation

Phylogenetic reconstruction based on the DNA sequences of up to four genes shows monophyly of the genus *Nannacara* (three species used in this study) and its sister relationship with the genus *Ivanacara* (one species present in our study). The monotypic genus *Cleithracara* (*C. maronii*) represents then basal lineage to the rest of *Nannacara* + *Ivanacara* (Fig. 2). The observed karyotype characteristics, i.e. the diploid chromosome number, the karyotype and the phenotype, were mapped on the phylogenetic tree and allowed reconstruction of the scenario of genome/karyotype evolution in the studied cichlids as well as to reconstruct as well as of the most likely hypothetical karyotype of an ancestor of the whole group. An ancestral karyotype of 2n = 48 was hypothesized as (16m-sm + 32 st-a) and was estimated as a basal stage for the clade by the most parsimonious reconstruction based on our material. The ancestor also had most likely only one pair of CMA_3 sites (Fig. 2).

Discussion

Cytogenetic characteristics

Two of the five species presented within this study have been previously studied in Thompson (1979), Marescalchi (2004) and reviewed in Feldberg et al. (2003). The

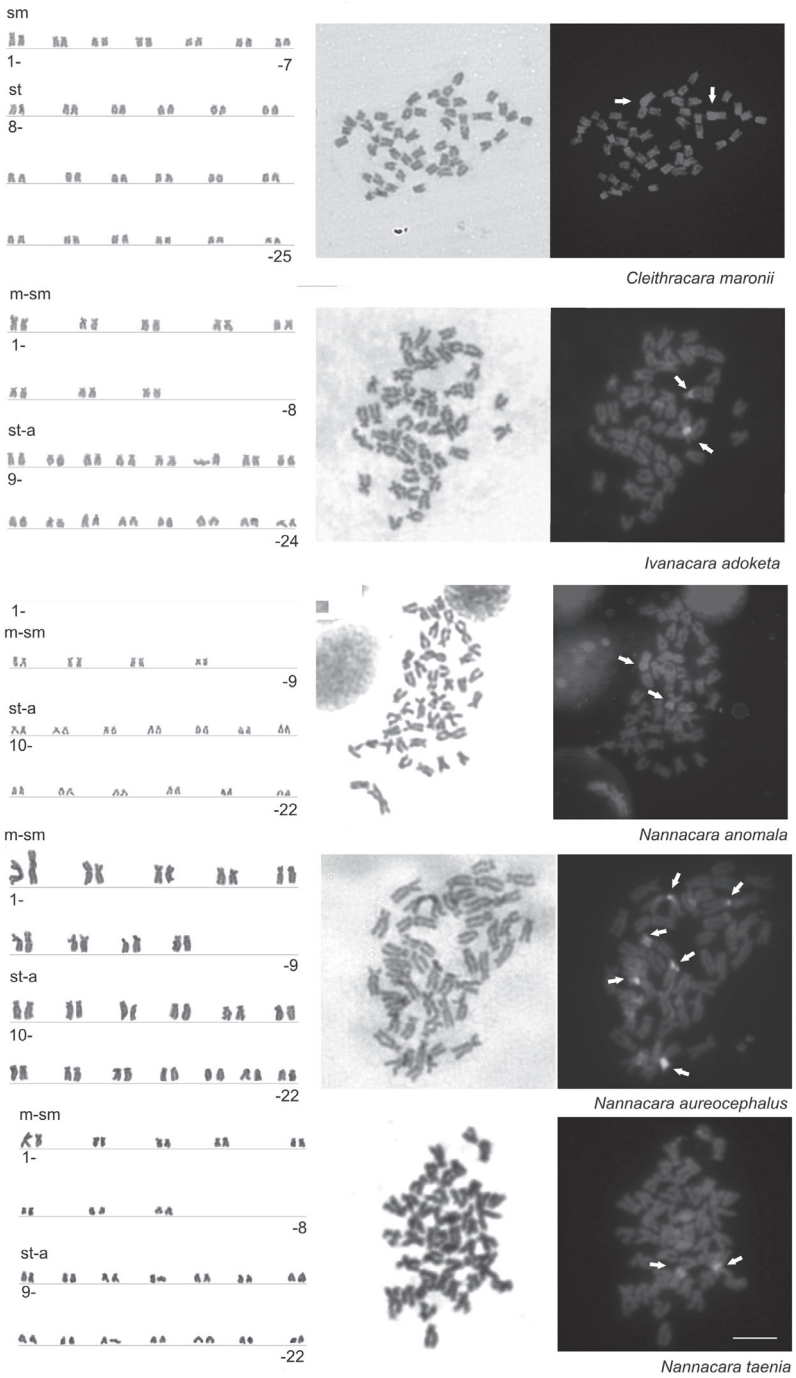

Figure 1. Karyotypes arranged from Giemsa stained chromosomes (left) of five species of cichlids: *C. maronii*, *I. adoketa*, *N. anomala*, *N. aureocephalus*, *N. taenia*. Selected metaphases stained with Giemsa staining (center) and sequentially by CDD banding (right). White arrows indicate chromosomes with positive Chromomycin A_3 signals. Bar=10μm.

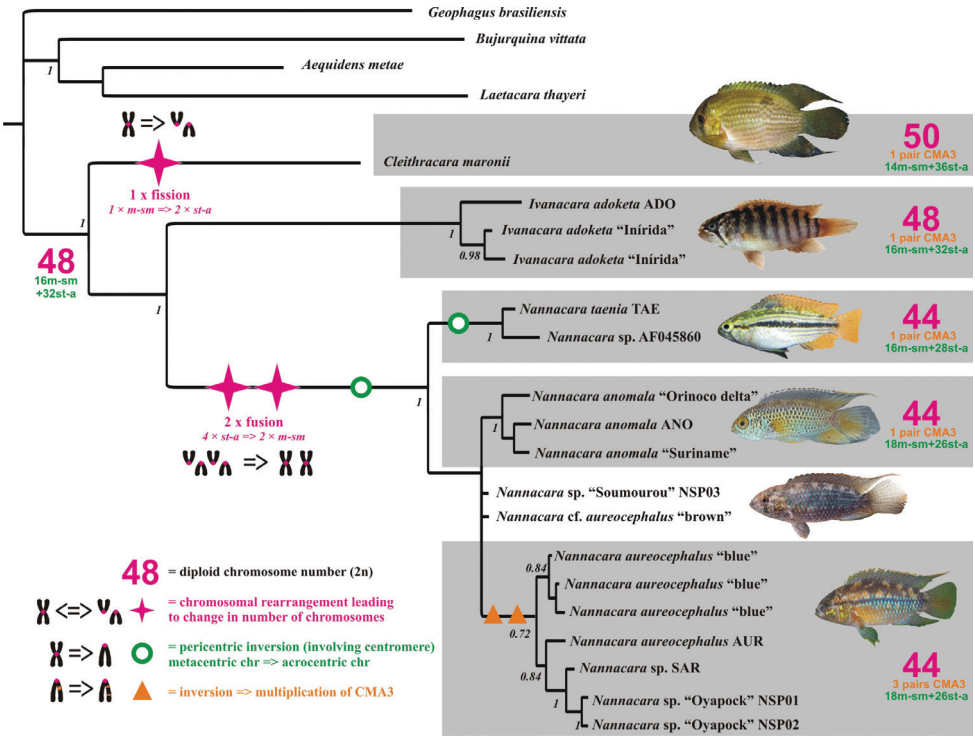

Figure 2. Phylogenetic relationships of cichlid fishes of genera *Nannacara*, *Ivanacara* and *Cleithracara*. Phylogenetic tree reconstructed based on the mitochondrial (cytochrome b, 16S rRNA) and nuclear (S7, RAG1) genes. Karyotype characteristics, such as diploid chromosomal number (2n), karyotype formula and CMA_3 phenotype were mapped on the tree and interpreted under the most parsimonious criterion. Ancestral karyotype of the group evolved from the ancestral cichlid karyotype 48st-a (Mank and Avise 2006) by increasing number of sub-metacentric chromosomes. One fission (in *Cleithracara* clade) and two fusion events (in the *Nannacara* clade) were detected, followed by at least one pericentric inversion in the latter case causing the decrease of the number of sub-metacentric chromosomes. Second pericentric inversion occurred in *N. taenia*, and another inversion leading to the multiplication of the CMA_3 regions occurred in *N. aureocephalus*.

karyotype of *Nannacara anomala* corresponds in both the chromosomal number (2n=44) and the karyotype (18m-sm+26st-a) to the results of Thompson (1979). The karyotype of *C. maronii* corresponds with various previous studies in chromosomal number (2n = 50; Marescalchi 2004, see Feldberg et al. 2003), but slightly differs in the karyotype description: while in our study we recognized seven pairs of sub-metacentric chromosomes (14m-sm+36st-a), Marescalchi (2004) found only six pairs of those. However, inspecting the study of Marescalchi (2004), we found one additional pair of sub-metacentric chromosomes in their original karyotype data as well, so it is fully comparable with our results.

In the clade of Neotropical cichlids, three trends in karyotype differentiation can be distinguished (Feldberg et al. 2003). First trend - also called Karyotype "A" by

Thompson (1979) – is characterized by maintaining the ancestral karyotype of 2n=48 with mostly subtelocentric-acrocentric elements (karyotype of 48st-a, although not exclusively) and evolved mostly by the pericentric inversions (during which the centromere is shifted from the central position of chromosome). Second evolutionary trend is similar to the previous one and additionally suppose the chromosomal breakage/fission events (Feldberg et al. 2003), leading to the increasing diploid chromosome number usually to the 2n=50 or 52, extremely up to 2n=60). This karyotype is dominated by uniarmed chromosomes. The third evolutionary trend - also called Karyotype "B" in Thompson (1979) – is represented by the opposite evolutionary scenario - mostly centric fusions played role in evolution from the ancestral karyotype, which lead to reduction of diploid chromosome number accompanied by increasing number of metacentric and submetacentric chromosomes (Thompson 1979, Poletto et al. 2010). This trend of chromosome number reduction seems to be parallel to some other fish groups like it was uncovered in killifishes (Cyprinodontiformes, Nothobranchiidae) Völker et al. (2008).

All of the species within the studied evolutionary lineage have a higher proportion of sub-metacentric chromosomes in their karyotypes compared with the rest of cichlids (Poletto et al. 2010). Especially considering the fact that the ancestral cichlid karyotype has been postulated as 2n=48 and 48st-a, i.e. no sub-metacentric chromosomes are present (Poletto et al. 2010), the whole *Nannacara – Ivanacara – Cleithracara* clade seems to have evolutionary derived karyotype within cichlids. Based on Thompson's (1979) classification, the whole lineage possess the karyotype type "B" characterized by higher proportion of the sub-metacentric chromosomes, although not all the species have the lower number of chromosomes then the ancestral stage, which is usually characteristic for the karyotype "B" as well (Thompson 1979). Interestingly, the chromosome rearrangements and formation of karyotype "B" occurred several times independently in cichlid evolution, as from 41 examined Neotropical cichlids, the karyotype "B" has been found in three unrelated lineages: in the species *Bujurquina vittata* (Heckel, 1840) (tribe Cichlasomatini), in the genus *Apistogramma* Regan, 1913 (tribe Geophagini) and in the genus *Symphysodon* Heckel, 1840 (tribe Heroini; sister tribe of Cichlasomatini; Thompson 1979). Strikingly, the most similar karyotype formula possessed by all the species of the genera *Apistogramma* (22-24m-sm+16-22st-a) and *Dicrossus* Steindachner, 1875 (12m-sm+34st-a), which also represent another two unrelated lineage of the dwarf cichlids (Thompson 1979, Feldberg et al. 2003), and then a few other species like *Cichlasoma paranaense* Kullander, 1983 (14-20m-sm+28-34st-a), *Mesonauta festivus/insignis* (Heckel, 1840) (12m-sm+36st-a), *Crenicichla niederleinii* (Holmberg, 1891) (14m-sm+34st-a) and *Astronotus ocellatus* (Agassiz, 1831) and *Astronotus crassipinnis* (Heckel, 1840) (12-18m-sm+30-36st-a, Feldberg et al. 2003). Note, that although the karyotype composed of mostly subtelocentric-acrocentric chromosomes is considered as ancestral for the cichlids, it is not generally ancestral trait for other fish groups. Therefore, the emergence of karyotype "B" (with more submetacentric chromosomes) probably represents secondary change back to the "common teleost karyotype" (Thompson 1979, Arai 2011).

CMA_3 patterns

The CMA_3 signals represent usually the GC-rich DNA segments of heterochromatic regions, often correlated with the location of active or inactive NORs, usually represented by the rDNA regions in genome (Schmid and Guttenbach 1988, Ráb et al. 1999, Poletto et al. 2010, but see Fontana et al. 2001, Gromicho et al. 2005 or Saitoh and Laemmli 1994). The number of CMA_3 signals found within this study corresponds to what has been previously observed in cichlids – i.e. the most common number of NORs in Neotropical cichlids is one pair, but in some species were found up to three pairs (Feldberg et al. 2003, Poletto et al. 2010). In the *Nannacara – Ivanacara – Cleithracara* clade, all species except for *N. aureocephalus* possess only one pair of CMA_3 signals in their karyotype. *N. aureocephalus* has three pairs of CMA_3 signals, which is usually interpreted as the result of inversion followed by the multiplication of the rDNA regions (Poletto et al. 2010). Further, one of the observed CMA_3 regions in this species is located in the centromeric region.

After Feldberg et al. (2003), one pair of NORs on the larger pair of chromosomes represents the most common NOR phenotype for the whole family Cichlidae. Further, Hsu et al. (1975) suggested that species with the single pair of NORs should be considered as more primitive that the karyotype with several NOR pairs hinting that the ancestral karyotypes possess less NORs than the evolutionary derived. Multiplication of NORs is usually caused by the chromosomal rearrangements, such as translocation or inversion but recently an increasing number of studies has shown the cases of rDNA multiplication caused by the activity of transposable elements.(Cioffi et al. 2010, Symonová et al. 2013, Schneider et al. 2013). As summarized in Feldberg et al. (2003), five out of 15 analysed species of the subfamily Cichlasomatinae (tribes Heroini + Cichlasomatini) possess multiple NOR pairs, i.e. *Caquetaia spectabilis* (Steindachner, 1875) (Feldberg et al. 2003), *Cichlasoma paranaense* Kullander, 1983 (Feldberg et al. 2003), *Mesonauata insignis* and *M. festivus* (Heckel, 1840) (Feldberg et al. 2003) and *Symphysodon aequifasciatus* Pellegrin, 1904 (Feldberg et al. 2003).

Phylogeny of *Nannacara – Ivanacara – Cleithracara* cichlids

The phylogenetic reconstruction of the *Nannacara – Ivanacara – Cleithracara* clade (also called NIC clade in Musilová et al. 2008, 2009) corresponds to the results observed in the previous studies (Musilová et al. 2008, 2009). This suggests the basal position of the monotypic genus *Cleithracara* followed by the *Ivanacara* (one species) sister to the rest of fishes from the genus *Nannacara* (three species). Within *Nannacara*, the *N. taenia* has basal position and *N. anomala* + *N. aureocephalus* represent the sister species. In this study, we did not include two species of the studied clade, i.e. *Nannacara quadrispinae* and *Ivanacara bimaculata*, which we failed to obtain either as live individuals for cytogenetics, or as samples for DNA analysis. Especially *I. bimaculata* would be crucial for confirmation of monophyly of the genus *Ivanacara,* since *I. bimaculata* was previously

found as closely related to the fishes of the genus *Nannacara* then to *I. adoketa* based on morphological data set (Musilová et al. 2009).

Within *N. aureocephalus*, more distinct forms are known; some of them were introduced into the aquarium trade under different names. So far no robust revision of *Nannacara* is available, and it is therefore difficult to make any taxonomic conclusion based on our data set. However, at least two different forms of *N. aureocephalus* are spread among the aquarium hobbyist within Central Europe (Germany, Poland, Czech Republic, Slovakia) – one of them called "blue" and the other one called "brown" both included in our analyses. These forms are not of artificial origin, as usually F1 progeny of the wild caught individuals has been studied. Intuitively, the blue morph shows more light-blue coloration with iridescent elements both on the face and body, while the "brown" form doesn't have the iridescent coloration and possess darker brown to dark-green coloration pattern. We have shown that those two morphs are genetically distinct; however, more detailed future work is necessary on this species/genus.

Karyotype differentiation

Cichlid karyotypes show some general common features - for example many species from African and Neotropical cichlids possess one pair of significantly larger chromosomes. Although the homology of the largest chromosome within the African lineage has been proved (Ferreira et al. 2010) as well as high synteny conservation of African cichlid genomes (Mazzuchelli et al. 2012), it is, however, not yet clear to what extent is the homology present across the whole family Cichlidae (Valente et al. 2009).

Although all the studied species from the *Nannacara – Ivanacara – Cleithracara* clade are characterized by the karyotype "B" (Thompson 1979), they underwent different evolutionary paths in past. The phylogenetic reconstruction of the karyotype evolution suggests the following scenario: from the ancestral karyotype, first the karyotype of the *Cleithracara maronii* (2n = 50; 14mt-sm + 36 st-a) evolved by fission event of one sub-metacentric chromosome pair, falling apart into two additional pairs of subtelocentric-acrocentric chromosomes. While the karyotype of *Ivanacara adoketa* remained unchanged compared with the ancestral one, in the lineage of *Nannacara*, two fusions occurred decreasing chromosomal number to 2n = 44. These fusions were followed by pericentric inversions, which again decreased the number of sub-metacentric chromosomes. At least one pericentric inversion happened in the base of all *Nannacara*, and additional pericentric inversion happened in the *N. taenia* lineage. Finally, two inversion impacting CMA_3 regions happened in *N. aureocephalus* leading to the multiplicaiton of these signals.

The proposed mechanisms of chromosomal rearrangements are described in cichlids as well as in other fish species. Usually the sub-metacentric chromosome arises during the (centric) fusion, when two acrocentric-telocentric chromosomes fuse (Thompson 1979). However, the number of sub-metacentric chromosomes in karyotype is not evolutionarily stable. The sub-metacentric chromosome changes back to the

acrocentric-subtelocentric chromosome by inversion, which involves the centromere, i.e. the pericentric inversion (Feldberg et al. 2003, Poletto et al. 2010). Further, those pericentric inversions are considered as the main mechanism generally contributing to changes in chromosome arms size in various percomorph lineages (Galetti et al. 2000, Affonso 2005). In general, the taxon sampling within such comparative studies is however still too low to be able to make a strong conclusion about the general trends in cichlid karyotype evolution (Feldberg et al. 2003, Poletto et al. 2010).

To conclude, we aimed to provide a comparative study on a small scale of three genera combining molecular and cytogenetic approaches. Assuming that cytogenetic data provide additional information, which is undetectable by molecular genetics (Ráb et al. 2007), we expected a broad insight into the genome evolution of the studied group. In the dwarf cichlid genus *Nannacara* and its relatives (*Ivanacara* and *Cleithracara*), we reconstructed the phylogeny and we found substantial amount of karyotype characteristics, which we were able to interpret in the evolutionary context.

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RESEARCH ARTICLE

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Possible interspecific origin of the B chromosome of *Hypsiboas albopunctatus* (Spix, 1824) (Anura, Hylidae), revealed by microdissection, chromosome painting, and reverse hybridisation

Simone Lilian Gruber[1], Débora Diniz[2], Patrícia Elda Sobrinho-Scudeler[3], Fausto Foresti[3], Célio Fernando Baptista Haddad[4], Sanae Kasahara[1]

1 *UNESP, Universidade Estadual Paulista, Instituto de Biociências, Departamento de Biologia, Av. 24A 1515, 13506-900, Rio Claro, SP, Brazil* **2** *UESB, Universidade Estadual do Sudoeste da Bahia, Departamento de Ciências Biológicas, Rua José Moreira Sobrinho s/n, Jequiezinho, 45206-190, Jequié, BA, Brazil* **3** *UNESP, Universidade Estadual Paulista, Instituto de Biociências, Departamento de Morfologia, Distrito de Rubião Júnior s/n, 18618-970, Botucatu, SP, Brazil* **4** *UNESP, Universidade Estadual Paulista, Instituto de Biociências, Departamento de Zoologia, Av. 24A 1515, 13506-900, Rio Claro, SP, Brazil*

Corresponding author: *Simone Lilian Gruber* (sisilgg@hotmail.com)

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Abstract

The B chromosome in the hylid *Hypsiboas albopunctatus* (2n = 22 + B) is small, almost entirely composed of C-positive heterochromatin, and does not pair with any chromosome of the A complement. B probe, obtained by microdissection and DOP-PCR amplification, was used to search for homology between the B and regular chromosomes of *H. albopunctatus* and of the related species *H. raniceps* (Cope, 1862). Reverse hybridisation was also carried out in the investigation. The B probe exclusively painted the supernumerary, not hybridising any other chromosomes in *H. albopunctatus*, but all *H. raniceps* chromosomes showed small labelling signals. This result might be an indication that differences exist between the repetitive sequences of A and B chromosomes of *H. albopunctatus*, and that the chromosomes of *H. raniceps* and the heterochromatin of the B chromosome of *H. albopunctatus* are enriched with the same type of repetitive DNA. In meiotic preparations, the B labelled about 30% of scored spermatids, revealing a non-mendelian inheritance, and the painted B in micronucleus suggests that the supernumerary is eliminated

from germ line cells. Although our results could suggest an interespecific origin of the B at first sight, further analysis on its repetitive sequences is still necessary. Nevertheless, the accumulation of repetitive sequences, detected in another species, even though closely related, remains an intriguing question.

Keywords
Supernumerary chromosome, FISH, GISH, replication banding, Hylidae family, heterochromatin, repetitive DNA

Introduction

B chromosomes are extra elements present in the genome of diverse groups of plants and animals (Jones and Rees 1982; Green 1990; Jones and Houben 2003; Camacho 2004, 2005; Schmid et al. 2010).With exception of dispensability for normal growth and development, many of the B characteristics, for example, numerical variability within and between individuals, smaller size than chromosomes from the A complement, heterochromatic nature, abnormal segregation at cell divisions, and non-mendelian inheritance, are not universal, indicating that the supernumerary chromosomes might correspond to distinct complex systems whose origins in the species are still the subject of extensive discussions.

To date, approximately 2% of karyotyped species of amphibians (Schmid et al. 2010; Green 2004) have shown B chromosomes, but among them only 16 anuran species were described with supernumeraries (Schmid et al. 2010; Green 2004; Milani et al. 2010). One of the most intriguing cases was reported in *Leiopelma hochstetteri* Fitzinger, 1861 from New Zealand, carrying two types of B chromosomes, one of them possibly related to a 0W/00 sex chromosomes system (Green 1988, 1991). Analyses on the molecular content of amphibian B chromosomes are scarce, and the only reported cases are in the frog *Leiopelma hochstetteri* (Sharbel et al. 1998) and in the salamander *Dicamptodon tenebrosus* (Baird & Girard, 1852) (Brinkman et al. 2000). In both cases, microdissected B chromosomes were analysed by Southern blotting, revealing that the B chromosomes share repetitive sequences with chromosomes of the A complement. The B chromosome of *Leiopelma hochstetteri* was also reported to contain specific sequences that were not present in any A chromosomes. Recently, B chromosomes of two species of Brazilian frogs, *Hypsiboas albopunctatus* and *Physalaemus feioi* Cassini, Cruz, & Caramaschi, 2010, were microdissected to generate probes for chromosome painting (Gruber et al. 2011; Campos et al. 2012).

Our previous analysis (Gruber et al. 2007) in the tree frog *H. albopunctatus* had shown one B chromosome in some individuals. This supernumerary was very similar in size and morphology to NOR-bearing chromosome 8, and had a large amount of late replicating C-positive heterochromatin, with a small bright DAPI region in the short arms. It was observed that the B was univalent, not pairing with any other chromosome in metaphase I cells. The present work was carried out to investigate whether the B of *H. albopunctatus* shares sequence homology with chromosomes of the A complement of the same species or of its closely related species *H. raniceps* which

could shed light upon questions about its intraspecific or interspecific origin. We used microdissection of B and chromosome painting (FISH), and additionally genomic hybridisation (GISH) with DNA of *H. raniceps* in the metaphases of *H. albopunctatus* harbouring one B chromosome.

Material and methods

In this study, we used chromosome preparations from fourmales of *Hypsiboas albopunctatus*, among which three had B chromosome, and from two females of *H. raniceps*:

H. albopunctatus: São Paulo: Rio Claro (22°25'20"S, 47°34'23"W), CFBH 28554-57 (males).

H. raniceps: Goiás: Ilha do Bananal (10°46'07.9"S 50°00'12.1"W), CFBH 7431 (female). Mato Grosso: Santa Rita doTrivelato (13°47'10.2"S, 55°13'18.0"W), CFBH 22456 (female).

Voucher specimens were deposited in the amphibian collection Célio F.B. Haddad (CFBH) housed in the Departamento de Zoologia, Instituto de Biociências, UNESP, Rio Claro, SP, Brazil.

Direct cytological suspensions of bone marrow, liver, and testes were obtained according to Baldisera Jr. et al. (1993). The specimens of *H. albopunctatus* were injected with 5-bromodoxiuridine (BrdU) before colchicine treatment (Silva et al. 2000). Slides were conventionally stained with Giemsa and those of *H. albopunctatus* with B chromosome were also submitted to FPG (Fluorochrome Plus Giemsa) technique (Dutrillaux and Couturier 1981; Matsuda and Chapman 1995) to differentiate replication banding. The chromosomal images were captured with an Olympus digital camera D71 using the DP Controller program. Bi-armed chromosomes were classified as metacentric, submetacentric, or subtelocentric (Green and Sessions 1991, 2007).

Microdissections of B chromosome were carried out according to Dinizet al. (2008) from metaphase I cells of two male specimens of *H. albopunctatus* with 2n = 22 + B. The specimens CFBH 28554 and CFBH 28557 were used to generate the B54 and B57 probes, respectively. A meiotic cell suspension was dropped onto a 24 mm × 60 mm coverslip and was immediately stained with 5% Giemsa in phosphate buffer, pH 6.8. Using a glass needle micromanipulator coupled in a Nikkon inverted microscope, four B chromosomes were microdissected and transferred to a microtube containing 10 μL DOP-PCR mix (1x Thermo Sequenase buffer reaction, 0.2 mMdNTP, 2 μM DOP primer - 5' CCG ACT CGA GNN NNN NAT GTG G 3' - (Telenius et al.1992), and ultra-pure water up to 10 μL). This procedure was repeated four times for each individual.

PCR reactions were performed using a Veritti Thermocycler (Applied Biosystems). Samples were heated at 90°C for 10 minutes and 4U. Thermo Sequenase enzyme (USB) was added. The initial amplification of microdissected products was performed using

RAMP-PCR with the following conditions: 94°C for 3 min; followed by 12 cycles of 94°C for 1 min 30 s; 37°C for 2 min increasing 0.2°C/s up to 72°C; 72°C for 2 min; followed by another 30 cycles of 94°C for 1 min 30 s; 62°C for 1 min; 72°C for 1 min 30 s.

After RAMP-PCR, a standard PCR was carried out to generate a probe stock. This reaction was comprised of: 1x PCR buffer; 2 mM $MgCl_2$; 0.02 mMdNTP; 0.7 mM DOP primer; 2.5 U Taq polymerase; 2 μL RAMP-PCR products; and ultra-pure water up to 25 μL. The following PCR conditions were used: 90°C for 3 min; followed by 30 cycles at 90°C for 1 min 30 s; 56°C for 1 min 30 s; 72°C for 1 min 30 s. Finally, a third PCR was performed to label the microdissection products. The PCR reaction was comprised of: 1× PCR buffer; 2 mM $MgCl_2$; 0.05 mMdATP; 0.05 mMdCTP; 0.05 mMdGTP; 0.035 mMdTTP; 0.015 mM labelled dUTP (Digoxigenin-11-dUTP, Roche); 0.7 μM DOP primer; 2.5 U Taq polymerase; 3 μL of the products from the second PCR; and ultra-pure water up to 25 μL. The amplification steps were the same as described for the previous reaction. After each PCR, electrophoresis of the amplified samples was performed using a 1% agarose gel to verify the sizes of the fragments. The fragments were 400–800 bp. Each labelled PCR product was precipitated in ethanol and re-suspended in 10 25 μL.

The B54 and B57 probes were used to perform FISH in mitotic and meiotic chromosomes. Firstly, the probes were tested on samples from the individuals they were generated from, i.e., B54 was tested on cytological preparations from CFBH 28554 and probe B57, on preparations from CFBH 28557. Next, cross-specimen chromosome painting was performed in which the B54 probe was used on mitotic preparations from CFBH 28557. The B54 probe was also used on chromosome preparations of *H. albopunctatus* specimen with no B chromosome (CFBH 28556), and on chromosome preparations of *H. raniceps* specimens from two distinct localities (CFBH 7431 and CFBH 22456).

All FISH experiments were performed according to Pinkel et al. (1986) with modifications. The B probe hybridisation procedures were performed with 77% stringency to prevent non-specific labelling. For the reverse hybridisation, the probe obtained from the genomic DNA of *H. raniceps* was marked with digoxigenin by nick translation and 50% stringency was used.

List of Abbreviations

2n: diploid number; DAPI: 4'-6-diamidino-2-phenylindole; DOP-PCR: degenerate oligonucleotide-primed polymerase chain reaction; FISH: fluorescent *insitu* hybridisation; NOR: nucleolar organiser region; PCR: polymerase chain reaction.

Results

Fig. 1a shows the karyotype of *H. albopunctatus* with 2n = 22 + B chromosome after standard staining, and Fig. 1b, after FPG technique differentiating very late replicat-

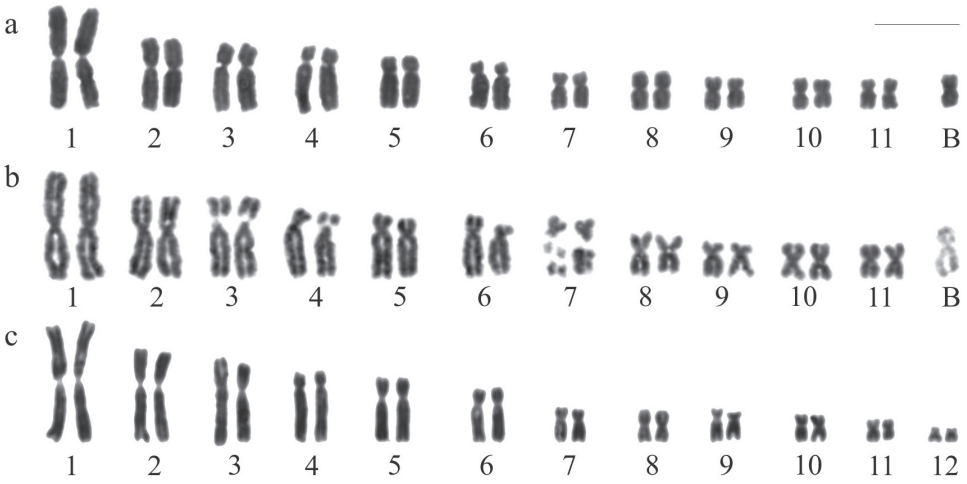

Figure 1. Karyotypes of *Hypsiboas*. **a–b** *H. albopunctatus*, male, 2n = 22 + B **c** *H. raniceps*, female, 2n = 24 **a, c** Giemsa stained karyotypes **b** replication bands. Bar = 10 mm.

ing regions in some chromosomes. The standard stained karyotype with 2n = 24 of *H. raniceps* is presented in Fig. 1c.

The probes B54 and B57 hybridised completely with the B in mitotic or meiotic preparations of the specimens they were generated from, i.e., the B54 probe painted the B chromosome in CFBH 28554 and the B57 probe painted the B chromosome in CFBH 28557. Fig. 2a shows a metaphase I cell of CFBH 28554 with DAPI staining and Fig. 2b the same cell with painted B54 probe. In Fig. 2c the painted B57 probe is shown in a metaphase II cell of CFBH 28557. Chromosomes of the A complement were not hybridised with any of the probes B54 and B57. The B54 probe cross-tested on mitotic preparation from the CFBH 28557 specimen also showed painting exclusively on the B chromosome (Fig. 2d). We performed a total of eight FISH experiments in which the B probes were tested on cells of individuals carrying the B chromosome, and identical results were always obtained. When the B54 probe was tested on mitotic preparation from *H. albopunctatus* without B chromosome (CFBH 28556) no labelling was observed in the chromosomes (figure not shown). The B54 probe tested on the meiotic preparations of the *H. albopunctatus* specimen CFBH 28554 with 2n = 22 + B showed that a micronucleus with the painted B probe was occasionally observed, close to an interphase nucleus (Fig. 2e). In the same cytological preparation, the B probe labelling was either present or absent in spermatids (Fig. 2f). The presence of B in spermatids was scored, showing B in 13% of the 66 analysed cells of one specimen, whereas in another, the B was observed in 31% of the 507 spermatids.

The hybridisation experiment with B probe on preparations of two *H. raniceps* specimens (CFBH 7431 and CFBH 22456), from different locations, showed numerous hybridisation signals interspersed throughout the chromosomes (Fig. 3a). The reverse hybridisation using gDNA of *H. raniceps* showed a small hybridisation signal

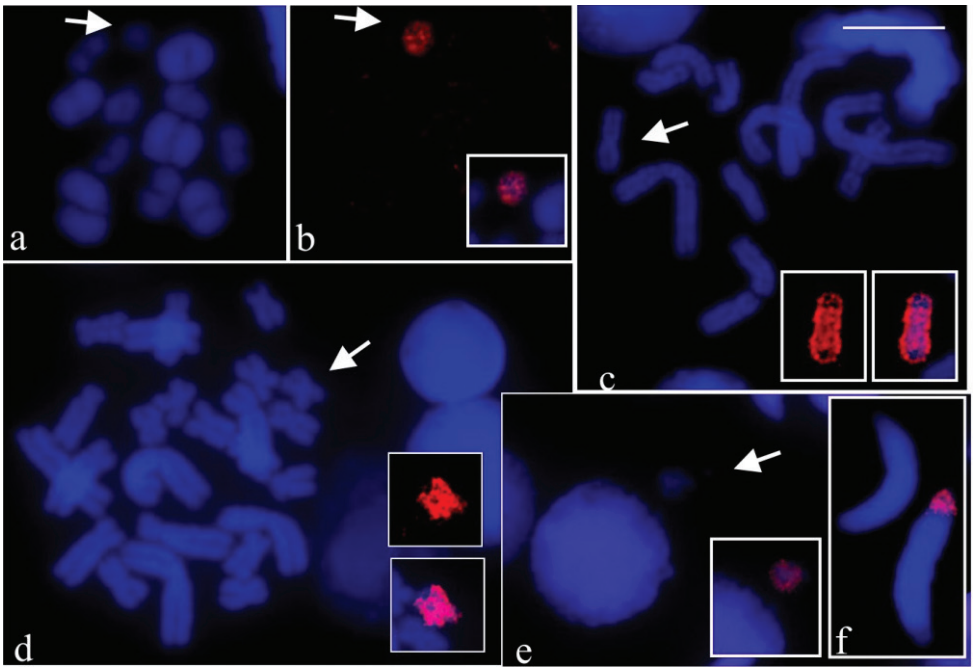

Figure 2. FISH using B probe in meiotic and mitotic preparations of *H. albopunctatus*. **a–b, e–f** *H. albopunctatus* (CFBH 28554), 2n = 22 + B; **c–d** *H. albopunctatus* (CFBH 28557), 2n = 22 + B **a** DAPI stained metaphase I **b** the same cell of **a** showing the B54 probe painting and the merged image of the B (inset) **c** DAPI stained metaphase II and B chromosome of same cell of **c** showing the B57 probe painting (inset), and merged images of the B (inset) **d** DAPI stained mitotic metaphase and B chromosome of same cell of **d**, showing the B54 probe painting (inset), and merged images of the B (inset) **e** DAPI stained interphase nucleus and micronucleus, and merged images showing B probe hybridisation on micronucleus (inset) **f** spermatids with and without labelling of B probe. Note in **a–d** completely labelled B chromosome (arrows). Bar = 10 mm.

on the supernumerary of metaphases from *H. albopunctatus* with B, and eventually, hybridisation signal was observed on some other chromosomes, like the 3 and 8 of A complement (Fig. 3b–d).

Discussion

The A chromosome complement of *H. albopunctatus* of the present study is in agreement with that previously reported (Cardoso 1986; Feitosa et al. 1995; Gruber et al. 2007; Ferro et al. 2012). One single B chromosome was observed in some of the Brazilian populations, whereas in Argentinian populations Ferro et al. (2012) identified two distinct morphological types of B, B1 and B2, with frequency varying from zero to three Bs per individual. The heterochromatin nature of the B was demonstrated by different cytogenetic techniques, including the here presented late replicating banding.

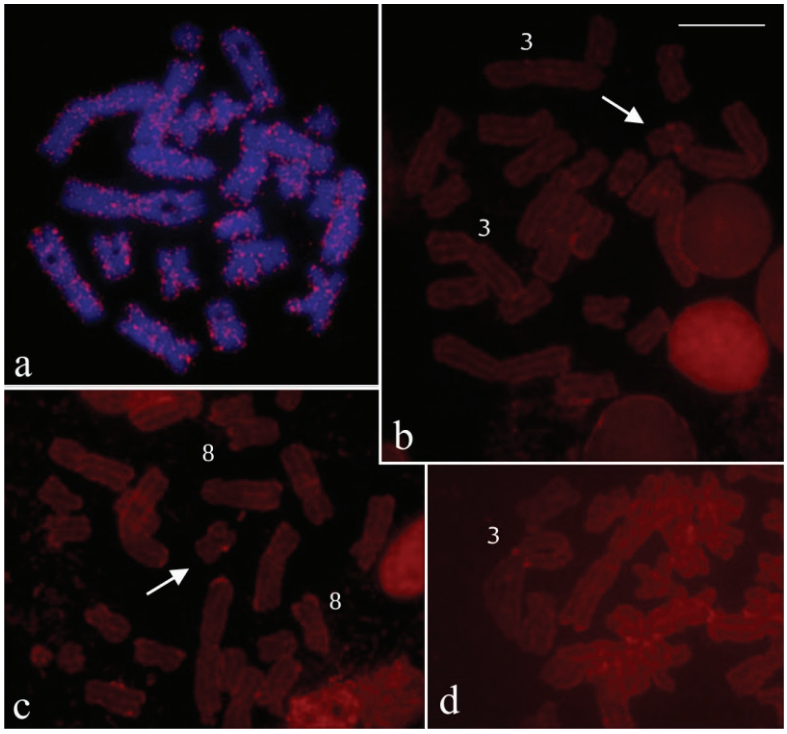

Figure 3. FISH using B probe in mitotic preparations of *Hypsiboas raniceps* and reverse hybridisation in *H. albopunctatus*. **a** *H. raniceps* (CFBH 7431), female, 2n=24 **b–c** *H. albopunctatus*, male, 2n = 22 +B (CFBH28557) **d** *H. albopunctatus*, male, 2n = 22 +B (CFBH28555) **a** chromosome painting with B54 probe, showing interspersed labelling in all chromosomes **b–d** reverse hybridisation withg gDNA of *H. raniceps* probe, showing labelling on B chromosome (arrow) and on chromosomes 3 and 8. Bar = 10 mm.

The B chromosome appeared with negative staining in both proximal short and long arms, excepting the terminal ends, and this result is in accordance with the C-positive staining observed only in the proximal arms (Gruber et al. 2007).

To obtain the supernumerary probe of *H. albopunctatus*, the microdissections performed in metaphase I cells, instead in mitotic metaphases, actually represented a good option. The univalent B was identified with certainty, preventing misidentification with the chromosomes 8. Besides, the smaller number of elements in the metaphase I meant better chromosome spreading and this avoided contamination during the scrapping procedure. The clear results of the FISH experiments indicate that the B chromosome probes were successfully generated, and the 77% of stringency prevented the probe hybridising non-specifically.

In all FISH experiments in which the B54 and B57 probes were tested in mitotic or meiotic cells of the specimens they were generated from, and in the case of cross-species, uniform and intense fluorescence was observed exclusively on the B chromosome. All these painting results are evidence that the B of *H. albopunctatus* harbours

a large amount of repetitive DNA sequences, a usual characteristic for the majority of the supernumeraries. Furthermore the identical results in the tests using probes obtained from different individuals indicate a significant degree of sequence conservation between the Bs in the studied population. The B probes did not paint any A chromosome in all experiments, including when tested in the *H. albopunctatus* specimen without supernumerary. The lack of hybridisation signal may suggest differences between the repetitive DNA sequences of the A and B chromosomes, unless the copy number of the B repetitive sequence on A chromosomes was too low to be detected by FISH. Another possibility is that sequences that are present in low copy number in B chromosome could be under amplified during the probe production, while the high repetitive sequences present in the heterochromatic blocks are overamplified. Although not frequent, findings where B contains sequences not shared with the chromosomes of A complement have been reported in some species, including the mammal *Nyctereutes procyonoides* (Gray, 1834) (Trifonov et al. 2002) and the fish *Alburnus alburnus* (Linnaeus, 1758)(Ziegler et al. 2003). In the vast majority of the cases, sequences found in the B are also shown in A chromosomes, as in the rodent *Apodemus peninsulae* (Thomas, 1907) (Karamysheva et al. 2002) and in the locust *Locusta migratoria* (Linnaeus, 1758) (Teruel et al. 2009). In the plant *Brachyscome dichromosomatica* Carter, 1978, bearing two types of supernumerary chromosomes (Leach et al. 1995; Houben et al. 1997; Houben et al. 2001), some of the repetitive sequences were found only in the B chromosomes, some were present in both B and A chromosomes, and others were present in the B or A chromosomes of other plants of the *Brachycome* genus.

In the investigations on the possible sequence homology of the supernumerary with chromosomes of the closely related *H. raniceps*, the FISH with B probe provided a peculiar painting pattern, but a technical artefact was discarded, since a reproducible pattern was obtained in large metaphase samples of two individuals from distinct localities, using hybridisations with 77% stringency. The observed pattern throughout the chromosomes closely resembles that shown in FISH experiments with retrotransposon probes (Meles et al. 2008; Cioffi et al. 2012), but if we are dealing with transposable elements should be investigated. The presence of transposons on B chromosome is not a novelty, since several studies reported that the Bs mostly contain distinct types of repetitive sequences, including transposable and retrotransposable elements, besides satellite DNA and rDNA (review in Camacho et al. 2005). It is surprising that the repetitive sequences found in B are absent on A chromosomes of *H. albopunctatus*, but are abundant in the chromosomes of *H. raniceps*. The reverse hybridisation confirms that some sequences are actually shared by the B and the regular chromosomes of *H. raniceps*. In *H. albopunctatus*, these sequences are accumulated in the heterochromatin of the B, whereas in *H. raniceps*, they are dispersed throughout the euchromatin of the chromosomes. The supernumerary of *H. albopunctatus* is heterogeneous in content, as it was revealed by replication bands and the reverse hybridisation, confirming our previous finding with C banding and DAPI staining (Gruber et al. 2007). One of the B repetitive sequences is shared with *H. raniceps* and seems to correspond to a transposable element.

The present analysis using B probe, allowing the precise identification of the B, confirmed it is a univalent, not pairing with any chromosome of the A complement in metaphase I cells of *H. albopunctatus*, and its occasional elimination as micronuclei in spermatogenic cells. The occurrence of the B only in about 29% of the 573 spermatids reinforced that the supernumerary segregation is lower than mendelian ratio, at least in males. May be this would explain the unaltered occurrence of one B per individual in the population of Rio Claro, from our first report until now. It is interesting to remark that the *H. albopunctatus* populations of Argentina presented accumulation mechanism, that is usually observed for Bs. While in this species the maximum number was three Bs per individual, in the hylid *Gastrotheca espeletia* Duellman & Hillis, 1987, Schmid et al. (2002) observed that the three types of B chromosomes were found from one up to nine Bs per individual. Certainly, further analyses are still recommended for better understanding of the B chromosome transmission during the meiosis in *H. albopunctatus*, and the possible absence of B accumulation during meiosis.

Based on our painting results, is very tempting to suggest an interespecific origin of the B, but this may be a premature conclusion, since the distribution of repetitive sequences on B and A chromosomes can be unequal (Montiel et al. 2012), and we should also consider that the dynamics of repetitive sequences suffers from the influence of diverse factors (Biémont and Vieira 2005). Furthermore, phylogenetic data of Faivovich et al. (2005), Wiens et al. (2010), and Pyron and Wiens (2011) show that, despite of the differences in the diploid number, *H. albopunctatus* and *H. raniceps* are in the same clade in the phylogenetic tree of *Hypsiboas* genus, and they are included in the same *H. albopunctatus* phenetic group. Therefore it is important to consider that both species are closely related and probably they inherited diverse types of DNA from a common ancestor. The origin of B chromosome of *H. albopunctatus* remains unsolved and approaches, like the investigation of whole sequencing of the supernumerary DNA, would provide further data to shed light on this question.

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Authors contributions

SLG performed the routine and molecular cytogenetic studies and drafted the manuscript. DD and PESS helped with microdissection and chromosome painting experiments and

revised the manuscript. FF supervised the microdissection and chromosome painting experiments and revised the manuscript. CFBH provided support on zoological information, carried out the species identification, and revised the manuscript. SK supervised the cytogenetic studies, participated in the draft, and in the revision of the final text. All authors read and approved the final manuscript.

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Genomic homeology between *Pennisetum purpureum* and *Pennisetum glaucum* (Poaceae)

Gabriela Barreto dos Reis[1], Amanda Teixeira Mesquita[1], Giovana Augusta Torres[1], Larissa Fonseca Andrade-Vieira[1], Antônio Vander Pereira[2], Lisete Chamma Davide[1]

1 *Departament of Biology, Federal University of Lavras, Zip Code 37200-000, Lavras, MG, Brazil* **2** *Laboratory of Plant Genetics , Brazilian Agricultural Research Corporation, Embrapa Dairy Cattle, Zip Code 36038-330, Juiz de Fora, MG, Brazil*

Corresponding author: *Lisete Chamma Davide* (lisete.ufla@gmail.com)

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Abstract

The genus *Pennisetum* (Richard, 1805) includes two economically important tropical forage plants: *Pennisetum purpureum* (Schumacher, 1827) (elephant grass), with 2n = 4x = 28 chromosomes and genomes A'A'BB, and *Pennisetum glaucum* (Linnaeus, 1753) (pearl millet), with 2n = 2x = 14 chromosomes and genomes AA. The genetic proximity between them allows hybrids to be obtained (2n = 3x = 21) that yield forage of higher quality in relation to the parents. The study of genomic relationships provides subsidies for the knowledge about phylogenetic relations and evolution, and is useful in breeding programs seeking gene introgression. Concerning elephant grass and pearl millet, the homeology between the genomes A and A', and between these and the genome B, has been reported by conventional cytogenetic techniques. The objective of the present study was to demonstrate the degree of homeology between these genomes by means of genomic *in situ* hybridization (GISH). The results confirmed the homeology between the genomes A of pearl millet and A'B of elephant grass, and showed that there are differences in the distribution and proportion of homologous regions after hybridization. Discussion regarding the evolutionary origin of *P. purpureum* and *P. glaucum* was also included.

Keywords

Homeology, *Pennisetum purpureum*, *Pennisetum glaucum*, Genomic *in situ* hybridization

Introduction

The genus *Pennisetum* (Richard, 1805) is one of the most important in family Poaceae family. It comprises about 140 species, distributed in five sections (*Penicillaria, Brevivalvula, Gymnothrix, Heterostachya* and *Eu-Pennisetum*) based on morphological characteristics (Stapf and Hubbard 1934). The section *Penicillaria* includes the economically most important species: elephant grass [*Pennisetum purpureum* (Schumacher, 1827)], used as forage, and pearl millet [*Pennisetum glaucum* (Linnaeus, 1753)], used as cereal and forage (Martel et al. 2004).

Molecular analyses based on mitochondrial DNA (Chowdhury and Smith 1988), chloroplast DNA (Renno et al. 2001) and repetitive DNA sequences (Ingham et al. 1993) have revealed significant relationship between the genomes of *P. glaucum, P. purpureum* and *Pennisteum squamulatum* Fresen., suggesting that these three species may have a common origin (Martel et al. 2004). Among these, the cultivated species *P. glaucum* and *P. purpureum* are phylogenetically related, possessing a close resemblance between their genomes, constituting a monophyletic group with recent divergence (Martel et al. 2004). In this sense, *P. glaucum* is an annual, alogamous, diploid species (2n = 2x = 14, genomes AA), with genome DNA content of 4.72 pg, and constitutes the primary genic pool of this genus. In turn, *P. purpureum* belongs to the secondary genic group and is a perennial, alogamous, tetraploid species (2n = 4x = 28, genomes A'A'BB), with genome DNA content of 4.60 pg (Martel et al. 1997). Both species have approximately the same DNA content (pg), but differ with regards to the monoploid size genome (basic number, x). *P. purpureum* is a tetraploid and have about half the DNA content (1.15 pg) of the *P. glaucum* monoploid genome (2.36 pg). Further, *P. pupureum* have smaller chromosomes than *P. glaucum* (Martel et al. 2004). This shows that important chromosome changes may be linked to the evolution and divergence among these species (Andrade-Vieira 2010; Barbosa et al. 2003; Martel et al. 2004; Robert et al. 2011).

In spite of their integrating distinct genic groups and differing as to ploidy level, the genetic proximity between these two species becomes evident when the occurrence of natural hybridization is observed. This sexual compatibility is partial, and results in sterile triploid hybrids (2n = 3x = 21, genome AA'B) (Hanna 1987; Martel et al. 2004; Robert et al. 2011; Techio et al. 2006). Cytologically, the genomic proximity has been demonstrated by meiotic analyses of triploids hybrids obtained in breeding programs. During diakinesis and metaphase I in this hybrid, the formation of seven bivalents is frequently observed, resulting from the pairing among chromosomes of genomes A and A' of *P. glaucum* and *P. purpureum*, respectively, as well as seven univalents of genome B of *P. purpureum* (Jauhar 1968, 1981; Pantulu 1967; Sree Rangasamy 1972; Techio et al. 2005, 2006). The occurrence, even at low frequency, of trivalents and numbers of bivalents above seven suggests both allo- as well as autosindetic pairing among the genomes A, A' and B (Sethi et al. 1970; Techio et al. 2005, 2006). These observations on the configurations of bivalents and univalents during meiosis, as well as the morphology of metaphase chromosomes, constitute the

pioneering works demonstrating homeology among the genomes A, A' and B. The obtained results suggest that the genome A of *P. glaucum* has larger homeology with genome A' of *P. purpureum*, whereas the origin of genome B has not been defined (Jauhar 1981; Techio et al. 2005, 2006).

In this sense, despite evidence for a common evolutionary origin between *P. glaucum* and *P. purpureum* and the economic importance of these species, there are no studies providing more conclusive data with respect to the homeology among genomes A, A' and B. Thus, the objective of this work is to describe the proportion and distribution of the homologous regions present in genomes A of *P. glaucum* and A'B of *P. purpureum*, by cytomolecular analyses using genomic *in situ* hybridization (GISH).

Material and methods

Plant material and genomic DNAs

The evaluations were carried out in mitotic metaphases of the parental *P. purpureum* (access BAG 65) and *P. glaucum* (access BN2), and of the triploid hybrid originating from this crossing (BAG 65 × BN2). The plant material and genomic DNAs were provided by the Active Germplasm Bank of Elephant Grass (BAGCE) from EMBRAPA Dairy Cattle (Brazilian Research Institute) and elephant grass breeding program, experimental field José Henrique Bruschi, municipality of Coronel Pacheco, Minas Gerais State, Brazil.

Chromosome preparation

Roots from seeds or cuttings of BAG 65, BN2 and triploid hybrid accession were collected and pretreated with a 12.5 $mg.L^{-1}$ cycloheximide : 150 $mg.L^{-1}$ 8-hydroxyquinoline solution for 2 h 45 min, at 4 °C, and fixed in ethanol : acetic acid solution (3:1), as proposed by Techio et al. (2002). Fixed root tips were digested with pectinase : cellulase (100U:200U) solution in citrate–phosphate buffer (pH 4.8) for 40 min (*P. glaucum*) and 3 h 30 min (*P. purpureum* and interspecific hybrid), at 37 °C, in moist chamber. Slides were prepared as proposed by Dong et al. (2000). A root tip was transferred to a slide and macerated with a drop of ethanol : acetic acid solution (2:1) using a fine-pointed forceps. The slide then was warmed over an alcohol flame. It could called flame-drying method.

Genomic *in situ* hybridization

Genomic DNAs of *P. glaucum* and *P. purpureum* were labeling with biotin-16-dUTP through nick-translation reaction method, thus yielding the genomic probes.

The hybridization technique was carried out according to Jiang et al. (1995). The hybridization mixture [55% formamide (v/v), 10% dextran sulfate (w/v), 2X SSC, pH=7.0, and 2 μL of probe marked with biotin] was denatured at 95 °C, for 8 min. Chromosome preparation was denatured with 70% formamide in 2X SSC (saline sodium citrate) at 85 °C, for 1 min 20 sec (Andrade-Vieira et al. 2013) and hybridized in the mixture at 37 °C for, at least, 16 h in a moist chamber. Detection of the probe marked with biotin was performed with streptavidin conjugated with Alexa Fluor® 488. Chromosomes were counterstained with 1 $\mu g.mL^{-1}$ 4',6-diamidino-2-phenylindole (DAPI) Vectashield® antifade solution (Vector Laboratories). The slides were evaluated under an epi-fluorescence Nikon Eclipse E600 microscope. Images of interest were digitized by means of a refrigerated monochromatic Nikon DSQi1MC camera, and processed using the software NIS-Element BR 4.00.03 (Nikon) and Adobe Photoshop CS3.

In order to evaluate the level of homeology between genomes A, A' and B, the chromosomes of five metaphases from each genome were measured, as well as the proportion occupied by the genomic probe, using the Image Tool 3.0 program. The obtained data were used to create karyograms for comparison of the evaluated genomes.

Results and discussion

Previous analyses of meiotic pairing in the triploid hybrid have showed that the genomes A and A′ are more related. On the other side, between both and the genome B there are affinity/homeology reduced (Jauhar and Hanna 1998, Techio et al. 2005). In this study, hybridization of genomic probes of *P. glaucum* and *P. purpureum* were used for the first time to demonstrate and to confirm homeology among the three genomes. It was evaluated the distribution and proportion of these homeologous regions in the family constituted by the parental *P. purpureum* (BAG 65) and *P. glaucum* (BN2), and by the triploid hybrid (BAG 65 × BN2) originating from this crossing.

The higher level of homeology between genomes A and A' was confirmed because the 14 chromosomes belonging to genome A' of *P. purpureum* were strongly marked and distinguished from the 14 chromosomes from genome B using the genomic DNA of *P. glaucum* (genome A) as probe in metaphases of *P. purpureum* (Fig. 1a). The chromosomes of genome A' presented marks in along almost role chromosome length, whereas genome B presented small marks dispersed over the length of its chromosomes (Fig. 2a). Moreover, approximately 29% of *P. purpureum* genome (A'B) was hybridized by the genome A of *P. glaucum* (Table 1). This percentual represents only the A' genome since the markers on genome B chromosomes were not record because it were dispersed on chromosomes. The observed homeology was only quantified in the genome A' of *P. purpureum*, due to the difficulty in measuring the small and dispersed marks found in genome B (Fig. 2a).

The homeology between genomes A and A' was confirmed by the extensive marking of *P. glaucum* chromosomes by the probe A'B of *P. purpureum*. All 14 chromosomes from genome A of *P. glaucum* were almost completely marked, with large blocks of probe sig-

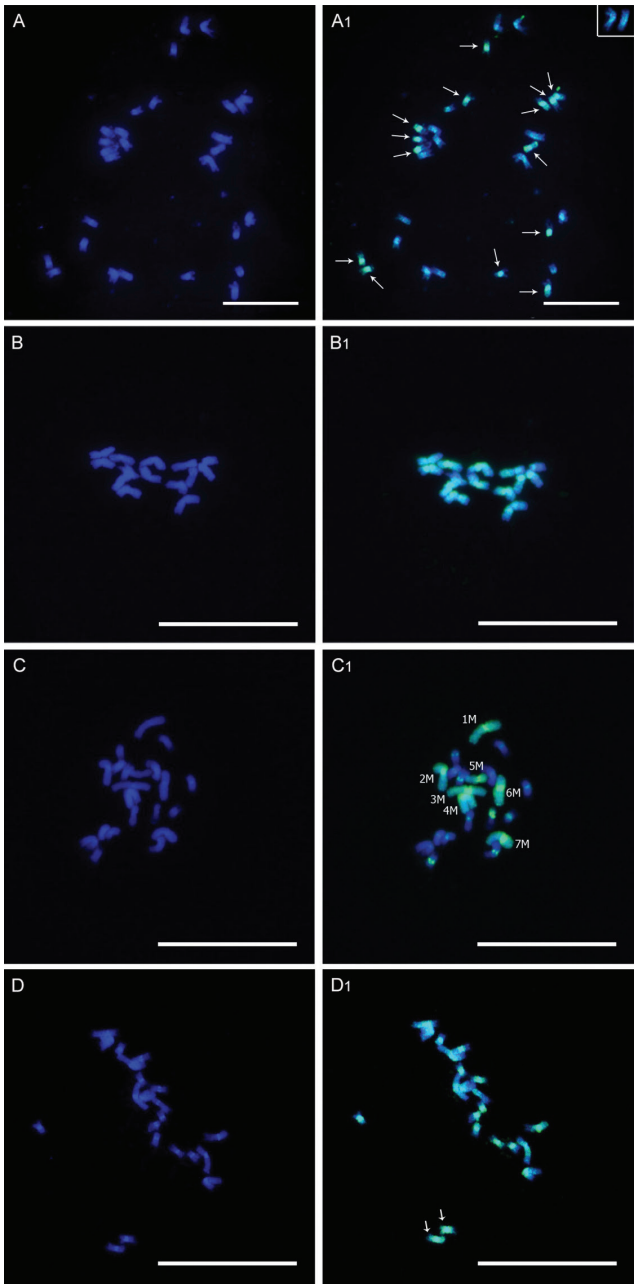

Figure 1. Metaphases of *Pennisetum purpureum (***A**), *Pennisetum glaucum* (**B**), and triploid hybrid (**C** and **D**). Chromosomes stained with DAPI (**A**, **B**, **C**, **D**) and probe markings in chromosomes indicated by green fluorescence (**A1**, **B1**, **C1**, **D1**). (**A1**) chromosomes of *P. purpureum* hybridized with genomic probe of *P. glaucum* (genome **A**), (**B1**) chromosome of *P. glaucum* hybridized with genomic probe of *P. purpureum* (genomes **A**'**B**), (**C1**) chromosomes of the triploid hybrid hybridized with genomic probe of *P. glaucum* (genome **A**), (**D1**) chromosomes of the triploid hybrid hybridized with genomic probe of *P. purpureum* (genomes **A**'**B**). Bar = 10 μm (**A**); Bar = 20 μm (**B**, **C** and **D**).

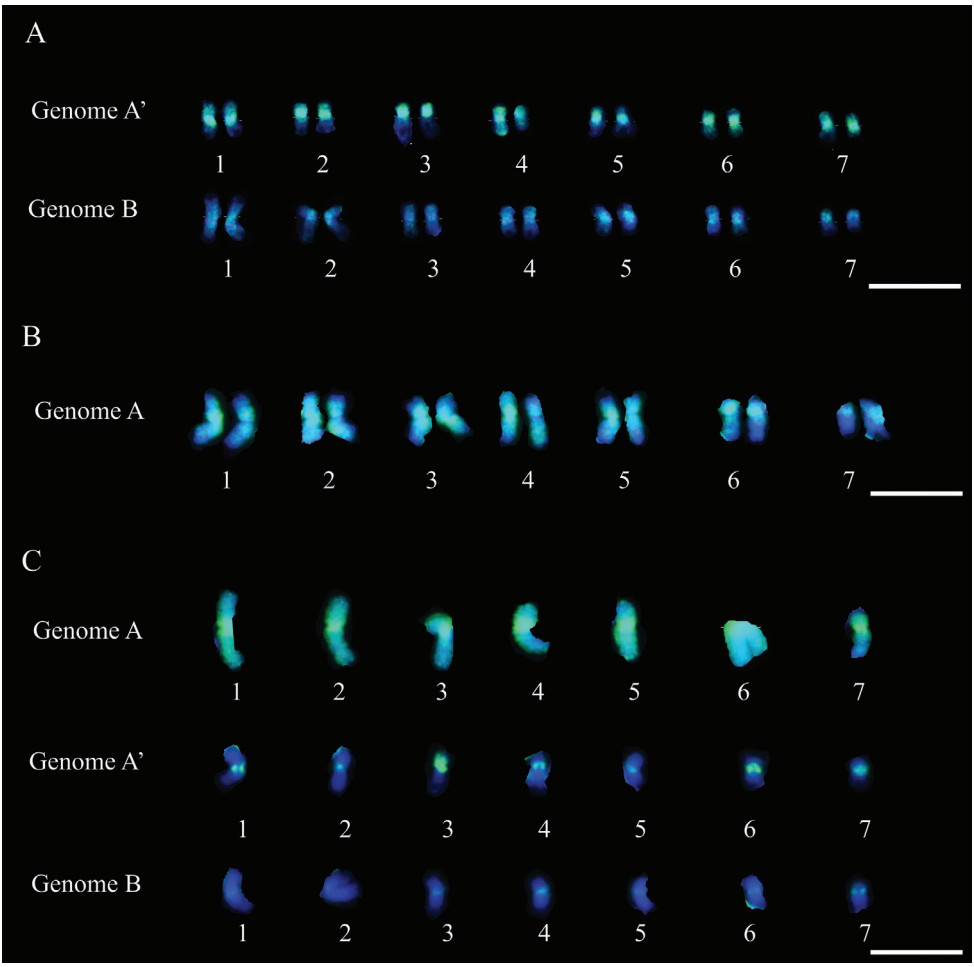

Figure 2. Karyograms of *Pennisetum purpureum* (**A**), *Pennisetum glaucum* (**B**) and triploid hybrid (**C**) identifying the chromosomes of genomes A, A 'and B in each genotype. Note that in (**A**) using genome A probe (*P. glaucum*), the chromosomes of genome A' were differed from chromosomes of genome B by the staining pattern. Genome A' chromosomes showed more apparent probe markings in green than genome B chromosomes. In (**B**), using the genome A'B probe (*P. purpureum*), all chromosomes were strongly labelled (markings in green). In (**C**), using the genome A probe (*P. glaucum*), the chromosomes of the A genome were fully labeled by the probe (markings in green), the genome A' were strongly marked in the centromeric region and the genome B, poorly marked. It also could be note the difference in the labeling pattern between the genome A probe on the chromosomes of genome A' in interspecific hybrid and parental *P. purpureum*. Bar = 10 μm.

nals observed on the chromosomes (Fig. 1b). The markings by the probe of genome A'B observed in the centromeric and pericentric regions represented 63% of the genome of *P. glaucum* (Table 1 and Fig. 2b). These marked portions result from hybridization, both between the genomes A and A' and, in smaller proportion, genomes A and B, observed both in karyograms of *P. purpureum* and triploid hybrids (Fig. 2a, c).

Table 1. Proportion of markings of genomic probes (A and A'B) on chromosomes of *Pennisetum purpureum*, *Pennisetum glaucum* and triploid hybrid.

Genotype	Total length of the chromosomes	Total length of the probe ***P. glaucum*** **(A)**	Total length of the probe ***P. pupureum*** **(A'B)**
P. purpureum	64,41	18,42 (28,60%)*	-
P. glaucum	59,01	-	37,29 (63,19%)
Triploid hybrid	73,95	40,06 (54,19%)	36,32 (49,13%)

* Proportion occupied by the probe for each genotype

In the triploid hybrid (AA'B) the hybridized portion of genome A (*P. glaucum*) corresponded to 54%, and the signal of probe A'B (*P. purpureum*) to 49% of its total genome (Table 1). Despite the similarity in proportion, the distribution pattern for the probes from the parental individuals was different in the hybrid (Fig 1c). The seven chromosome of the hybrid were entirely marked with the probe of genomic DNA from *P. glaucum*. The remaining chromosomes from *P. purpureum* parental (genome A´B) presented marks only in the centromeric and pericentromeric regions (Fig. 2c). However, when the probe with DNA of *P. purpureum* was used in chromosomes of the triploid hybrid the marks were observed mainly in centromeric and pericentromeric regions, but some chromosomes appearing almost totally marked (Fig. 1d).

The differences in marking pattern observed in the triploid hybrid, mainly between genomes A and A', could be explained by the presence of two genomes (A' and B) in the same probe. The observations evidence the changes arising from interspecific hybridization in *P. purpureum* genomes. Once combined in a polyploid hybrid nucleus, extensive reorganization may rapidly occur in the parental diploid genomes, both intra and intergenomically (Soltis and Soltis 1999; Chen et al. 2006). The rapid intergenomic rearrangements in polyploids in relation to the diploid progenitors have been demonstrated in allohexaploid F1 hybrids of *Avena sativa* (Leitch and Bennett 1997), in hexaploids of wheat (Nelson et al. 1995) and soybean (Shoemaker 1996), as well as in triploid hybrids embryos of *Pennisetum* (Campos 2007).

Besides the existing homeology among genomes A, A' and B, the utilization of GISH in the genomes of *P. glaucum*, *P. purpureum* and interspecific hybrid enable to verify the differences in chromosomes size, and also chromosomes number of these species. Analyzing cells of the interspecific hybrid, the difference in size between the parental chromosomes becomes evident, with those of *P. glaucum* being larger (Fig. 2c). It can also be observed that the total length of *P. purpureum* chromosomes did not increase proportionally in relation to those of *P. glaucum* (Table 1 and Fig 2a and b), and that the chromosomes of genome B do not differ significantly in size in relation to genome A' (Table 2 and Fig. 2a). These differences in size and chromosome number between the two species reflect their evolutionary history.

The evolutionary tendency among true grasses, which have a common and recent origin, is that the most derived species have emerged after reduction of the number and increase of the size of chromosomes in relation to the ancestors (Avdulov 1931;

Table 2. Total length (μm) of monoploid complement in genomes A, A' and B for each genotype.

Genotype	Total length of the genome A	Total length of the genome A'	Total length of the genome B
P. purpureum	-	23,8	23,95
P. glaucum	29,51	-	-
Triploid hybrid	17,63	10,54	12,44

Bennetzen 2007; Crepet and Feldman 1991; Martel et al. 2004; Paterson et al. 2004; Stebbins 1956, 1971). This tendency applies to the genus *Pennisetum*, whose common ancestral pattern presented the basic number of chromosomes x = 9. Furthermore, the evolutionary pattern inside the genus follows the same tendency, this way, it is observed that the ancestor of the species from section *Pennicillaria*, to which *P. purpureum* and *P. glaucum* belong, presented basic number of chromosomes x = 7. In this sense, analyzing the phylogeny of the genus *Pennisetum* presented by Martel et al. (2004) and the information on chromosome size and homeology of genomes A, A' and B observed in the present work, it can be inferred that the species *P. purpureum* and *P. glaucum* have concomitantly diverged from the common ancestor. The origin of *P. purpureum* occurred at the interspecific hybridization event, combining the genome A of the ancestor with genome B of a second, still unknown. Therefore, genome A' could be considered a subgenome of the ancestor A due to genomic and structural changes that occurred during evolution. On the other hand, the species from the primary genic pool of *P. glaucum* have diverged from the common ancestor through increase of chromosome size, probably by increment in the genic sequences. The increase in chromosome size in this species could be explained, as described by Poncet et al. (2002), by duplication of some genes as consequence of domestication syndrome. The presence of non-homologous recombination observed among the chromosomes of *P. glaucum* reinforces the hypothesis of genic duplication as part of the differentiation in this species (Jauhar 1970; Martel et al. 2004).

This hypothesis, presented for the evolution and divergence of *P. purpureum* and *P. glaucum* from the common ancestor, may be further reinforced by the differences observed in relation to the size of chromosomes from genomes A, A' and B, as shown in Table 2. Analyzing the size of the monoploid complement of genome A, it can be verified that it is 24% larger in relation to the length of the chromosomes of genome A' of *P. purpureum*. Considering that the genomes A and A' have evolved from an ancestor genome A, the difference in chromosome size could be related to genic duplication in *P. glaucum* and to genomic rearrangements observed in the allotetraploid hybrid *P. purpureum*. Rearrangements and loss of genomic sequences are common events after hybridization (Kellis et al. 2004), as observed in this study by comparison between different genomes combined in the triploid hybrid (Table 2). In this case, a reduction of 60% of genome A can be observed in the hybrid in relation to the parental *P. glaucum*, along with 44% and 52% reduction of genome A' and B, respectively, in relation to the parental *P. purpureum*.

In this work, GISH confirmed the homeology among genomes A, A' and B and enabled the identification and distribution of the homeologous regions in the chromosomes. Moreover, the GISH markings were able to separate the different genomes, leading the comparison on the size of the chromosomes in each of these three genomes. This distinction of the different genomes confirmed the occurrence of rearrangements after interspecific hybridization, especially when the synthetic triploid hybrid was analyzed, and prove the allotetraploid origin of *P. pupureum.* It also show that genomes A' and B have chromosomes similar in size. In evolutionary terms, the results reinforce that the genomes A and A' have diverged from an ancestral genome A by increase of chromosome size in *P. glaucum* and rearrangements and/or deletions in *P. purpureum*. The reorganizations occurring in the ancestral genome A during evolution have generated the subgenome A' of *P. purpureum*.

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RESEARCH ARTICLE

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Basic cytogenetics and physical mapping of 5S and 18S ribosomal genes in *Hoplias malabaricus* (Osteichthyes, Characiformes, Erythrinidae) from isolated natural lagoons: a conserved karyomorph along the Iguaçu river basin

Gisele Gemi[1], Roberto Laridondo Lui[1], Fernando Rodrigo Treco[2], Leonardo Marcel Paiz[3], Rafaela Maria Moresco[1], Vladimir Pavan Margarido[1]

1 *Centro de Ciências Biológicas e da Saúde, Universidade Estadual do Oeste do Paraná, Rua Universitária 2069, CEP: 85819-110, Cascavel, PR, Brazil* **2** *Universidade Paranaense, Avenida Julio Assis Cavalheiro 2000, CEP: 85601-000, Francisco Beltrão, PR, Brazil* **3** *Universidade Estadual de Maringá, Departamento de Biologia, CEP: 87020-900 Maringá, PR, Brazil*

Corresponding author: *Vladimir Pavan Margarido* (Vladimir.Margarido@unioeste.br)

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Abstract

Erythrinidae include Neotropical teleost fish that are widely distributed in South America. *Hoplias* Gill, 1903 include two large groups: *H. malabaricus* Bloch, 1794 and *H. lacerdae* Miranda Ribeiro, 1908. *Hoplias malabaricus* is characterized by remarkable karyotype diversity, with some karyomorphs widely distributed geographically while others are more restricted to certain river basins. Cytogenetic analyzes were performed in a population of *Hoplias malabaricus* from the Wildlife Refuge of Campos de Palmas, the Iguaçu River basin. The specimens showed diploid number of 42 chromosomes (24m+18sm) without differentiated sex chromosomes system. The impregnation by silver nitrate showed multiple AgNORs. Seven pairs (4, 7, 10, 13, 16, 20 and 21) carrying 18S rDNA were detected by FISH. Heterochromatin was verified in the centromeric and pericentromeric region of most chromosomes and the terminal region of some pairs. FISH with 5S rDNA probes showed two chromosome pairs carrying these sites in the interstitial region (8 and 14). The data obtained in this study are similar to those found for two other

populations of *H. malabaricus* already studied in the basin of the Iguaçu River, confirming the hypothesis that this species is natural, not having been introduced, as well as having an intrinsic characteristic, such as the largest number of sites of 18S rDNA.

Keywords
Chromosomal conservadorism, double-FISH, evolution, karyotype, rDNA

Introduction

The basin of the Iguaçu River, located in the southern region of the State of Paraná, is comprises a drainage area of 69,373 square km and a length of 1,275 km in its main riverbed. Its springs emerge from Serra do Mar and flow towards the First Plateau, or Plateau of Curitiba, and to the Second and Third Plateau. In the latter, the Iguaçu river basin is bordered by the Plateau of Palmas at the border of the State of Santa Catarina (Silva et al. 2001), where 79% belongs to the State of Paraná, 19% to the State of Santa Catarina and 2% to Argentina (Agostinho et al. 1997). The Iguaçu river basin has a low diversity of species and a high degree of endemism, with a total of 106 species, being 35 of Characiform, 46 of Siluriform and 11 Perciform (Baumgartner et al. 2012). This endemism has as its main cause the appearance of the Iguaçu Falls at the last part of the flow (Agostinho et al. 2004). In addition to this large geographical barrier of 72 meters, other barriers that segment the Iguaçu river were observed along its flow: Salto Caiacanga (9 meters), Salto Grande (13 meters), Salto Santiago (40 meters) and Salto Osorio (30 meters) (Maack 1981).

Erythrinidae are characterized by a sedentary lifestyle which consequently reduces gene flow between the populations that inhabit the same basin since they do not overcome obstacles, such as waterfalls (Blanco et al. 2010). This family is composed of three genera: *Erythrinus* Scopoli, 1777, *Hoplerythrinus* Gill, 1896 and *Hoplias* Gill, 1903 (Gayet et al. 2003). *Erythrinus* is comprised of two species, *E. erythrinus* Bloch & Schneider, 1801 and *E. kesslerie* Steindachner, 1877 (Oyakawa 2003). *Hoplerythrinus* includes three species: *H. cinereus* Gill, 1858, *H. gronovii* Valenciennes, 1847 and *H. unitaeniatus* Spix & Agassiz (Froese and Pauly 2014). *Hoplias* is the most widespread in South America, composed of two large groups: *Hoplias lacerdae* Miranda Ribeiro, 1908 and *Hoplias malabaricus* Bloch, 1794, the first group containing six species (Oyakawa and Mattox 2009), and the second is a classic case of cryptic species related to chromosomal aspects (Bertollo et al. 2000).

According to Bertollo et al. (2000), *Hoplias malabaricus* is a Neotropical freshwater species widely distributed and with great karyotype diversity (different karyomorph). The chromosomal studies show diversity in diploid number from 39 to 42 chromosomes, differences in chromosomal formulas and presence (karyomorph B, D and G) or absence (karyomorph A, C, E and F) of a sex chromosome system. According to this author, *H. malabaricus* includes seven karyomorph, being some of them more widely distributed, such as karyomorph A, C and F, and other restricted to only one or a few sites, such as karyomorph B, D, E and F. Populations of this species from the basin

of the Iguaçu river were previously analyzed by cytogenetic methods and two of these karyomorph were detected (A and B) (Lemos et al. 2002, Vicari et al. 2003, Vicari et al. 2006). The karyomorph A appears to be more widely distributed throughout this basin, while the karyomorph B is restricted to only one population in a region next to its riverbed side (Lemos et al. 2002).

With regards to the occurrence of *H. malabaricus* in the basin of the Iguaçu river, there is a controversy as to its origin in this location. According to Garavello et al. (1997), *H. malabaricus* would not be a native species of the Iguaçu river, which may have been introduced from nearby basins. Subsequently, a study with chromosomal populations of this species suggested that the karyomorph A is a native form of the Iguaçu River. In addition, the little karyotype diversity detected among the populations that were analyzed must be due to vicariant events (Vicari et al. 2006).

In this sense, the objective of this work was to study - through cytogenetic techniques - a population of *Hoplias malabaricus* collected in a natural lagoon in the region of Palmas, in the far south of the State of Paraná – Brazil. This lagoon has no contact with other aquatic environments and is isolated from other river systems, in order to better understand the geographical distribution of the group in the basin of the Iguaçu river.

Methods

Four specimens were collected (2 males and 2 females) of *Hoplias malabaricus* from isolated lagoons in the region of Palmas of the Wildlife Refuge of Campos de Palmas, in the Iguaçu river basin, belonging to the State of Paraná – Brazil (Fig. 1). This reduced sample is duet to the collections being made on a conservation unit, and a major sampling would be justified if intra- or interpopulational chromosomal polymorphisms were observed. The samples were anesthetized and sacrificed by an overdose of clove oil (Griffiths 2000) for the removal of the material for the cytogenetic study. The mitotic chromosomes were obtained from a cell suspension using the anterior portion of the kidney in accordance with the technique adapted by Bertollo et al. (1978) and Foresti et al. (1993). Thirty metaphases spreads from each fish were analyzed and ten of the best mitotic metaphases were used to measure karyotypes. For the AgNORs analysis, the impregnation by silver nitrate has been used based on the methodology of Howell and Black (1980), and to determine the distribution pattern of heterochromatin, C-banding with barium hydroxide was used, following the proposal of Sumner (1972) with modifications proposed by Lui et al. (2012). For the analysis of fluorescent *in situ* hybridization (FISH) 5S rDNA probes of *Leporinus elongatus* Valenciennes, 1850 (Martins et al. 2000) and 18S rDNA of *Prochilodus argenteus* Spix & Agassiz, 1829 were used (Hatanaka and Galetti 2004). Each one of them was marked, respectively, with digoxigenin-11-dUTP and biotin-16-dUTP (Roche). The detection and amplification of the hybridization signal was performed using antidigoxigenin-rhodamine for 5S rDNA (Roche) and avidin-FITC and anti-avidin-biotin for 18S rDNA (Sigma). FISH was performed according to Pinkel et al. (1986) and modifications suggested by

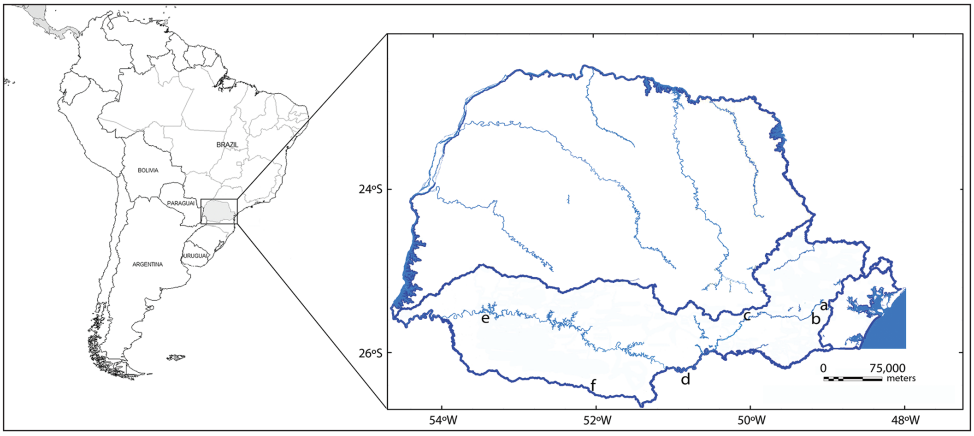

Figure 1. Map of sampling sites of *Hoplias malabaricus* populations in Iguaçu river basin: **a** Piraquara **b** São José dos Pinhais **c** Palmeira **d** Poço Preto **e** Nova Prata do Iguaçu and **f** Palmas (present paper).

Margarido and Moreira-Filho (2008). The best metaphases were captured in an Olympus BX60 photomicroscope with a digital camera DP71 and DPcontroller 3.2.1.276 software (Olympus). The FISH slides were analyzed with an epifluorescence photomicroscope under an appropriate filter. The chromosomes were arranged in groups classified in metacentric, submetacentric, subtelocentric and acrocentric, according to the calculation of arm ratio as proposed by Levan et al. (1964).

Results

The cytogenetic analysis observed diploid number of 42 chromosomes with 24 metacentric chromosomes and 18 submetacentric chromosomes, for male and female, and without a sex chromosome system (Fig. 2). The impregnation by silver nitrate showed multiple AgNORs, ranging from 4 to 6 NORs. The analyzed metaphases with silver nitrate impregnation presented bi-telomeric labels in the metacentric pair 7 and telomeric labels on the short arm of the metacentric pair 10 (Fig. 2, in box), coinciding with 18S rDNA, evidenced in FISH (Fig. 3). Five other pairs carrying rDNA 18S were marked by FISH, the metacentric 4 in both telomeric regions, the submetacentric pair 13 in the telomeric region of the short arm, pair 16 in the interstitial region of the long arm, pair 20 in both telomeric regions, and pair 21 in the terminal region of the long arm (Fig. 3). The C-banding revealed heterochromatin in the centromeric and pericentromeric region in most chromosomes of the complement, as well as bitelomeric and terminal heterochromatin in some chromosomes, these being coincident with the AgNORs (pairs 7 and 10) (Fig. 2). The FISH with 5S rDNA probe revealed two pairs of chromosomes, being interstitial on the long arm of the metacentric 8 and on the short arm close to the centromere of the submetacentric 14 (Fig. 3).

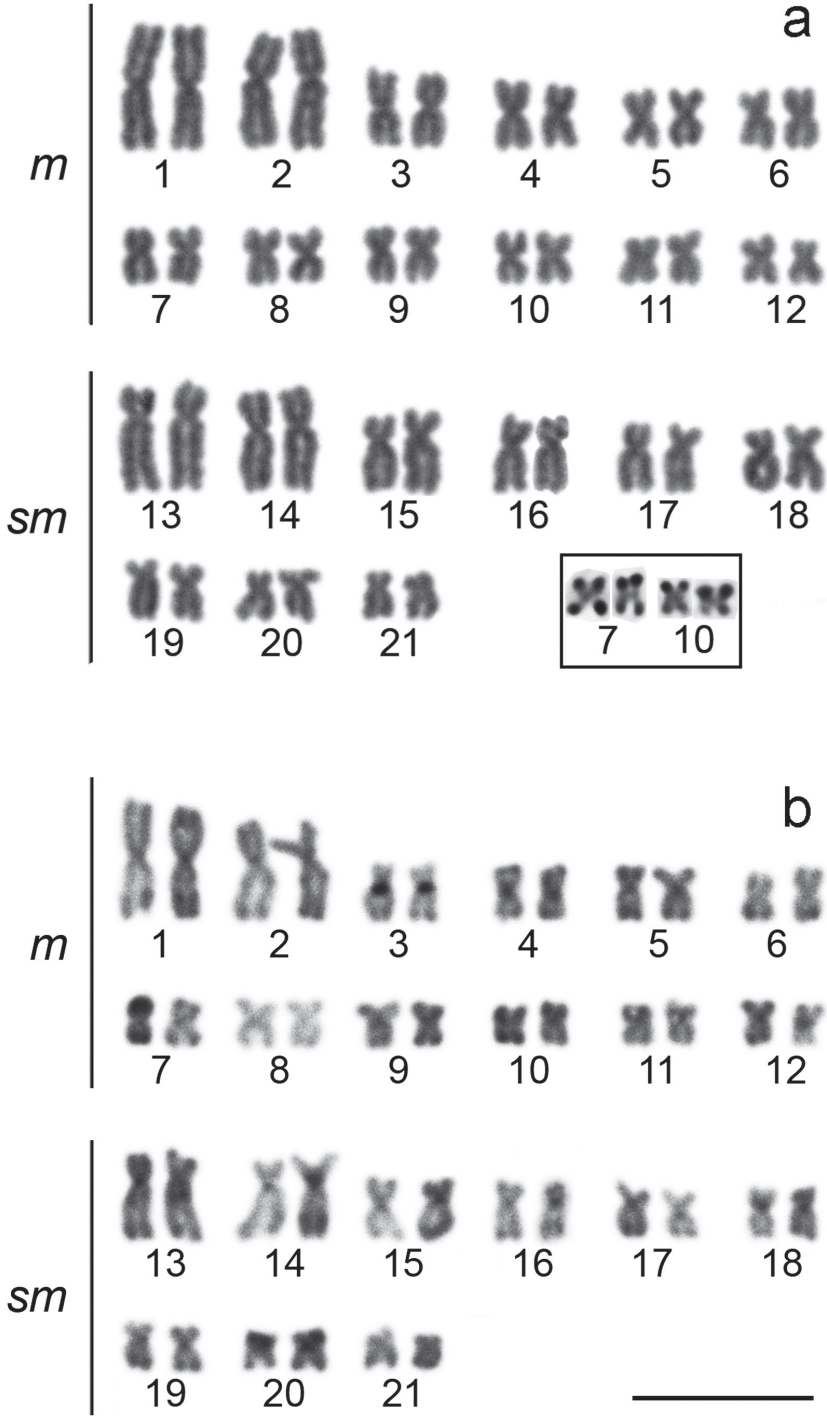

Figure 2. Karyotypes of *Hoplias malabaricus* stained with Giemsa (**a**) and treated through the C-banding (**b**). The AgNORs bearing chromosome pairs (7 and 10) are presented in box. Bar = 10 μm.

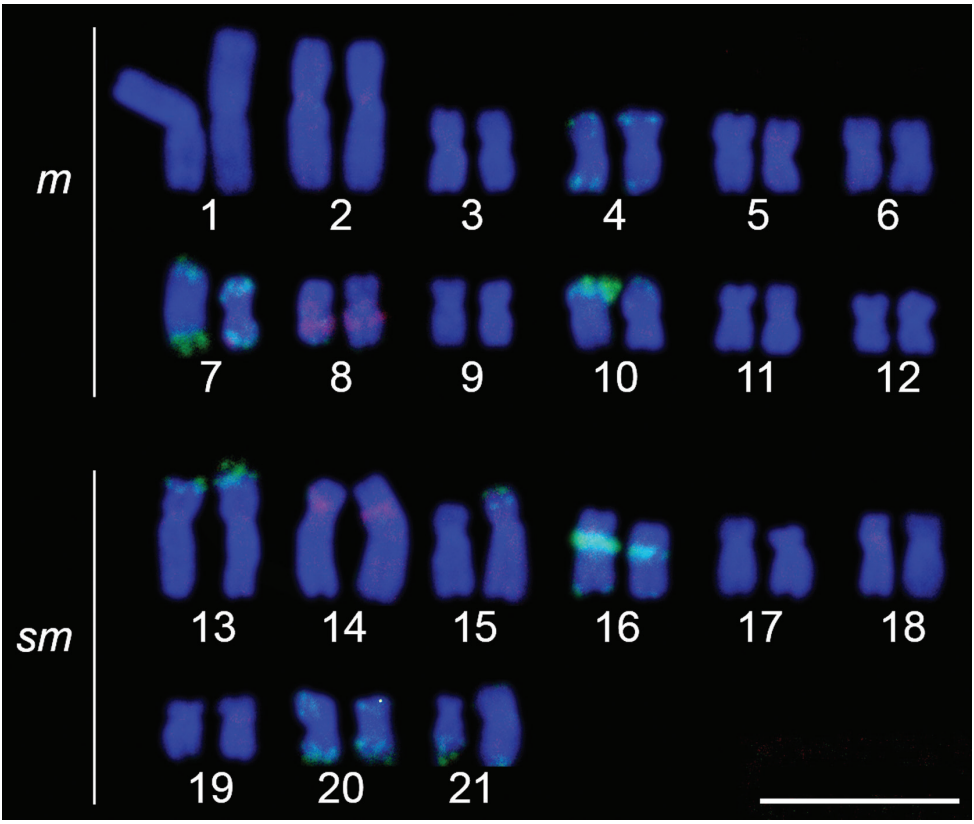

Figure 3. Karyotype of *Hoplias malabaricus* hybridized with 5S rDNA (digoxigenin, red) and 18S rDNA (FITC, green) probes. Bar = 10 μm.

Discussion

Hoplias malabaricus comprises a complex of species due to its wide karyotype diversity, and some karyomorphs are geographically widely distributed, while others have lower distribution and are restricted to certain basins, and even sympatric karyomorphs may occur without the detection of hybrids (Bertollo et al. 2000, Born and Bertollo 2006). The specimens analyzed showed chromosomal characteristics related to a diploid number, absence of a system of sexual chromosomes and karyotype formula that fits them in the karyomorph A of the *H. malabaricus* group as designated by Bertollo et al. (2000). Previous studies in populations of *H. malabaricus* from the Iguaçu river showed that this karyomorph is the most widely distributed in the basin (Fig. 1, Table 1). According to Blanco et al. (2010), who reviewed the chromosomal studies related to karyomorph A, a great part of South American river basins contain them, and multiple levels of chromosomal differentiation can be observed among allopatric populations (i.e., karyotype formula, heterochromatic distribution, AgNORs/18S rDNA, 5S rDNA and *5SHindIII* satellite DNA).

Table 1. Cytogenetical data of *Hoplias malabaricus* populations from Iguaçu river basin.

Locality	Karyomorph	Karyotype formula	AgNORs	Heterochromatin (C-banding)	18S rDNA	5S rDNA	Reference
Piraquara municipality (PR)	A	20m+22sm	Multiple: - 2 to 6 chromosomes (1 bitelomeric pair)	Pericentromeric and interstitial	-	-	1
São José dos Pinhais municipality (PR)	B	24m+16sm+2st (XX/XY)	Multiple	Pericentromeric	-	-	1
Poço Preto municipality (SC)	A	42 m-sm	-	-	-	-	2
Palmas municipality (PR)	A	24m+18sm	Multiple: - pair 7, m, bitel - pair 10, m, tel, sa	Pericentromeric and terminal	- pair 4, m, bitel - pair 7, m, bitel - pair 10, m, tel, sa - pair 13, sm, tel, sa - pair 16, sm, int, la - pair 20, sm, bitel - pair 21, sm, tel, la	- pair 8, m, int, la - pair 14, sm, int, sa	3
Nova Prata do Iguaçu municipality (PR)	A	24m+18sm	Multiple: - 3 to 8 chromosomes	Pericentromeric and terminal	-	-	4
Palmeira municipality (PR)	A	24m+18sm	Multiple (2 to 7 chromosomes): - pair 10, m, bitel - pair 16, sm, int, la - pair 21, sm, tel, la	Pericentromeric and terminal	- pair 4, m, tel, la - pair 10, m, bitel - pair 16, sm, int, la - pair 21, sm, tel, la	-	4, 5, 6

PR: Paraná state, Brazil; SC: Santa Catarina state, Brazil; m: metacentric; sm: submetacentric; tel: telomeric; bitel: bitelomeric; int: interstitial; la: long arm; sa: short arm.
References: 1 - Lemos et al. (2002); 2 - Bertollo et al. (2000); 3 - Present paper; 4 - Vicari et al. (2006); 5 - Vicari et al. (2003); 6 - Vicari et al. (2005)

For the populations of karyomorph A of *Hoplias malabaricus* from the Iguaçu river, the karyotype formula does not show any clear marker to differentiate populations throughout this basin (Vicari et al. 2006) or to distinguish them from populations in neighboring basins (Ribera, Tibagi and Ivai) of the Iguaçu river (Vicari et al. 2005), which is different from what is observed for allopatric populations of several other regions (Blanco et al. 2010). Despite the paper of Lemos et al. (2002), bringing a slightly distinct formula (20m + 22sm), and the one from Bertollo et al. (2000) of not separating meta- and submetacentric chromosomes, our observation of these karyotypes suggests that they could be rearranged by 24 metacentrics and 18 submetacentrics, as detected by Vicari et al. (2003, 2005, 2006) and for the population of this study. It is worth noting that this conservation of the karyotype formula is not a common situation for populations distributed along this basin, as was already observed for *Astyanax altiparanae* Garutti & Britski, 2000, *Oligosarcus longirostris* Menezes & Géry, 1983, *Corydoras paleatus* Jenyns, 1842, *Pimelodus ortmanni* Haseman, 1911 and *Glanidium ribeiroi* Haseman, 1911 (Kantek et al. 2007). Furthermore, in relation to karyomorph A of *H. malabaricus*, this level of conservation was not observed with other chromosomal markers.

The distribution of heterochromatin in all the karyomorphs of the *H. malabaricus* complex has often been described in the terminal and pericentromeric region of some pairs of chromosomes (Dergam and Bertollo 1990, Haff et al. 1993, Bertollo et al. 1997a, 1997b, Born and Bertollo 2000, Vicari et al. 2005, Blanco et al. 2010), and was also observed in the population of the present study. However, a small variation in the amount and location of heterochromatin can be observed between the various allopatric populations already studied (i.e., Blanco et al. 2010). When the distribution of heterochromatin of the population in this study is compared to others of the Iguaçu river (Lemos et al. 2002, Vicari et al. 2006), or even with those that are present in the basins next to the Iguaçu (Vicari et al. 2005), a great similarity can be observed.

The analyses carried out by Vicari et al. (2006) in populations belonging to the basin of the Iguaçu river (Nova Prata do Iguaçu and Palmeira), demonstrated a variable number of AgNORs, usually located in the telomeric region, and bitelomeric AgNORs were also found in both populations, as for the population analyzed in this study. However, interstitial nucleolus organizing regions were observed on the long arm of chromosome pair 16 of the Palmeira population, this characteristic being uncommon for *H. malabaricus*. Note that bitelomeric AgNORs have usually been found in *H. malabaricus* (Bertollo 1996, Born and Bertollo 2001), being this characteristic considered a probable synapomorphy for the group (Vicari et al. 2006). Up to now, there is no evidence of populations belonging to karyomorph A of *H. malabaricus* that do not have bitelomeric AgNORs (Blanco et al. 2010).

Only a population with hybridization data with 18S rDNA is described in literature regarding the Iguaçu river, with four pairs being detected, one pair with bitelomeric marking, one interstitial pair and two pairs with terminal marking. All these pairs of the previous study (Vicari et al. 2003, 2005) have chromosomes corresponding to the population of this study. In addition, other pairs showed sites carrying 18S rDNA (pair 4 and 20, bitelomeric; pair 13, terminal on short arm). More than one chromo-

some pair with 18S rDNA has already been detected for the karyomorph A of *H. malabaricus* (Blanco et al. 2010). However, this is the first report of this last three pairs.

This study showed two pairs of 5S rDNA sites carrying chromosomes. Previous studies showed that this marker varies in number of sites among populations of karyomorph A, with a small metacentric pair with interstitial marking that seems to be conserved (Ferreira et al. 2007, Blanco et al. 2010), and a second pair (large submetacentric) that can be detected with interstitial marking on the short arm in a population of the Sao Francisco river (Blanco et al. 2010). The two pairs detected in the population of the Iguaçu River in this paper seem to be corresponding to these pairs mentioned above. Ferreira et al. (2007) compared the location of 5S rDNA sites in three karyomorphs of the *H. malabaricus* group (A, D and F) and observed obvious differences between them, indicating that the number and distribution of sites are good markers of the Erythrinidae family, which shows the need for data for this marker in other populations of this basin, since the 5S rDNA data presented in this paper are the first related to the Iguaçu river basin.

With the uplift of the Iguaçu Falls, an effective geographic isolation was created for the ichthyofauna of the First and Second Plateaus in the largest part of the Iguaçu river (Maack 1981), resulting in a pronounced endemism of its ichthyofauna (Garavello et al. 1997). This endemism is proposed for several groups of fish, therefore the occurrence of *H. malabaricus* in the basin could be due to human introduction (Sampaio 1988, Dergam et al. 1998). However, other explanations are considered as well relating to its presence in the basin. The karyomorph A of *H. malabaricus* features a wide distribution throughout the southeast and south of Brazil, being present in several rivers in the State of Paraná, reaching Uruguay and Argentina (Bertollo et al. 2000). Due to the old shaping of the Iguaçu river basin and the broad distribution that has been detected for this karyomorph A, Vicari et al. (2006) proposed that this species may not have been introduced as was previously believed. The analysis of this population present in the city of Palmas reinforces this hypothesis, not only due to being another population of this karyomorph in the basin, but mainly because this population comes from a natural lagoon located in an isolated region that is part of the hydrographic system of the Iguaçu river.

Therefore, the population analyzed in this study showed the same diploid number, karyotype formula, lack of a differentiated sex chromosomes system when compared to other populations of the Iguaçu river, in addition to sharing some characteristics with respect to the number and location of AgNORs, distribution of heterochromatin and 18S rDNA sites. These data confirm the hypothesis that *H. malabaricus* is natural to the Iguaçu River, and in spite of presenting some intrinsic characteristics of this population, it represents the same evolutionary unit along the basin, which is in the process of allopatric differentiation through the setting of small rearrangements in the microstructure.

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RESEARCH ARTICLE

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Cytogenetics of *Melitoma segmentaria* (Fabricius, 1804) (Hymenoptera, Apidae) reveals differences in the characteristics of heterochromatin in bees

Maykon Passos Cristiano[1,2], Talitta Guimarães Simões[2], Denilce Meneses Lopes[2], Silvia das Graças Pompolo[2]

1 *Departamento de Biodiversidade, Evolução e Meio Ambiente, Universidade Federal de Ouro Preto, Morro do Cruzeiro, Ouro Preto, Minas Gerais, Brazil, 35400-000* **2** *Departamento de Biologia Geral, Universidade Federal de Viçosa, Avenida Peter Henry Rolfs s/n, Viçosa, Minas Gerais, Brazil, 36570-000*

Corresponding author: *Maykon Passos Cristiano* (maykoncristiano@hotmail.com)

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Abstract

To date, more than 65 species of Brazilian bees (of the superfamily Apoidea) have been cytogenetically studied, but only a few solitary species have been analyzed. One example is the genus *Melitoma* Lepeletier & Serville, 1828, for which there is no report in the literature with regard to cytogenetic studies. The objective of the present study is to analyze the chromosome number and morphology of the species *Melitoma segmentaria* (Fabricius, 1804), as well as to determine the pattern of heterochromatin distribution and identify the adenine–thymine (AT)- and guanine–cytosine (GC)-rich regions. *Melitoma segmentaria* presents chromosome numbers of 2n=30 (females) and n=15 (males). With C-banding, it is possible to classify the chromosomes into seven pseudo-acrocentric pairs (A^{M}), seven pseudo-acrocentric pairs with interstitial heterochromatin (A^{Mi}), and one totally heterochromatic metacentric pair (M^{h}). Fluorochrome staining has revealed that heterochromatin present in the chromosomal arms is rich in GC base pairs (CMA_3^+) and the centromeric region is rich in AT base pairs ($DAPI^+$). The composition found for *Melitoma* diverges from the pattern observed in other bees, in which the heterochromatin is usually rich in AT. In bees, few heterochromatic regions are rich in GC and these are usually associated with or localized close to the nucleolus organizer regions (NORs). Silver nitrate impregnation marks the heterochromatin present in the chromosome arms, which makes identification of the NOR in the chromosomes impossible. As this technique reveals proteins in the NOR, the observation that is made in the present study suggests that the proteins found in the heterochromatin are qualitatively similar to those in the NOR.

Keywords
cytogenetic characterization, heterochromatin, fluorochromes, solitary bees, karyotype evolution

Introduction

The genus *Melitoma* Lepeletier & Serville, 1828 belongs to the tribe Emphorini and has 10 described species. These are solitary bees, which nest in cavities in the soil, are typically gregarious, and are distributed from Mexico to Argentina (Mamede-Filho et al. 1991). These bees are closely associated with a particular plant, *Ipomoea* sp. (Convolvulaceae), and the presence or absence of this plant generally defines their distribution (Schlising 1970).

Cytogenetic studies of Brazilian bees are more common in the eusocial species belonging to the tribe Meliponini. These studies were initiated by Kerr (1948). Since then, more than 28 genera and 65 species have been analyzed. The haploid chromosome number in the bees of this tribe ranges from eight to eighteen, where n=17 is the predominant number (Rocha et al. 2003).

Little cytogenetic information has been obtained for solitary bees. In the literature, only some cytogenetic information for the species of the genus *Eufriesea* Cockerell, 1908 (Gomes et al. 1998), *Euglossa* Latreille, 1802 (Maffei et al. 2001, Fernandes et al. 2013), *Ceratina* Latreille, 1802, *Xylocopa* Latreille, 1802, and *Pithitis* Klug, 1807 (Hoshiba and Imai 1993) is found. The same is true for the genus *Melitoma*, where none of the ten species are cytogenetically characterized.

Cytogenetic studies are important because they contribute a great deal to the understanding of evolutionary mechanisms that contribute to the changes in genome organization. By using different chromosomal banding techniques we can study different chromosomal characters that can be used to solve taxonomic issues. A simple karyotype analysis allows the observation of variations, such as, differences in chromosome number, size, and specific base pair composition of the DNA, enhancing our knowledge of the evolution and phylogenetic relationships of different species (White 1973, Imai et al. 1994, Sumner 2003).

The "minimum interaction hypothesis" proposed by Imai et al. (1988), is the most commonly used mechanism to explain chromosome diversity and evolution in Hymenoptera, mainly in ants and bees (Rocha et al. 2003). According to this hypothesis, karyotype evolution is biased toward an increase in acrocentric chromosomes, thereby reducing the risk of deleterious rearrangements, due to a decrease of the potential contact among the chromosomes in the nucleus. Although occasional fusions that decrease the chromosome number are not excluded by "the minimum interaction hypothesis", fissions appear more likely. However, Fernandes et al. (2013) and Cardoso et al. (2014) based on the studies of solitary bees and ants, have suggested that other mechanisms may be involved in the karyotype evolution of social Hymenoptera.

In this context, the aim of the present study is to analyze the karyotype, including the chromosome number and morphology, distribution pattern of the heterochromatin, and richness of composition of the AT and GC base pairs, of the solitary bee species *M. segmentaria,* thereby contributing to an increase in the cytogenetic knowledge of this genus and providing interesting new insights about the genome organization in these bees.

Material and methods

To perform the cytogenetic study, 10 larvae of *M. segmentaria* within the nest cells were collected in Viçosa – Minas Gerais, Brazil (20°44'58.03"S, 42°51'8.98"W). We sampled 10 individual nests. The cells were opened in the laboratory to verify the larval stage. The larvae that were not at the post-defecation stage were maintained in a biological oxygen demand (BOD) chamber (Marconi, model MA-415/S), at 25ºC, until they reached the appropriate stage.

The metaphase chromosomes were obtained from the larval cerebral ganglia in the post-defecation stage (Imai et al. 1988). If the ganglia were large enough, they were divided into two or more sections. Chromosome characterization was performed by conventional Giemsa staining and C-banding (BSG method: Barium hydroxide (5%)/saline solution (2XSSC, pH 7.0)/Giemsa (8%)), as reported by Sumner (1972). Sequential staining with fluorochromes chromomycin A_3 (CMA_3) and 4',6-diamidino-2-phenylindole (DAPI) was carried out according to the methodology of Schweizer (1980). The technique Ag-NOR presented by Howell and Black (1980), was used for the location of the NOR.

The metaphases were analyzed with the aid of an Olympus BX 60 microscope coupled to an image capturing system, Q Color3 Olympus®. For analysis of the fluorochromes, WB filters (450 – 480 nm) were used for CMA_3 and WU filters (330 – 385 nm) for DAPI. The karyotypes were assembled according to the classification established by Imai (1991), which took into consideration the heterochromatin pattern.

Results and discussion

The species *M. segmentaria* showed a chromosome number of 2n=30 for females and n=15 for males (Fig. 1). This chromosome number was similar to that observed in other solitary bee species, including *Ceratina megastigmata* Yasumatsu and Hirashima, 1969 (2n=34), *Xylocopa appendiculata* Smith, 1952 (2n=32), and *Pithitis smaragdula* (Fabricius, 1787) (2n=28) (Hoshiba and Imai 1993). However, it was lower than the value found in *Euglossa*, that is, 2n=42 (Fernandes et al. 2013).

The C-banding technique allows the observation of large positive heterochromatic blocks in the chromosomes of *M. segmentaria* (Fig. 1c), wherein, at least one of the arms, has been completely heterochromatic. Taking into account the C-banding pat-

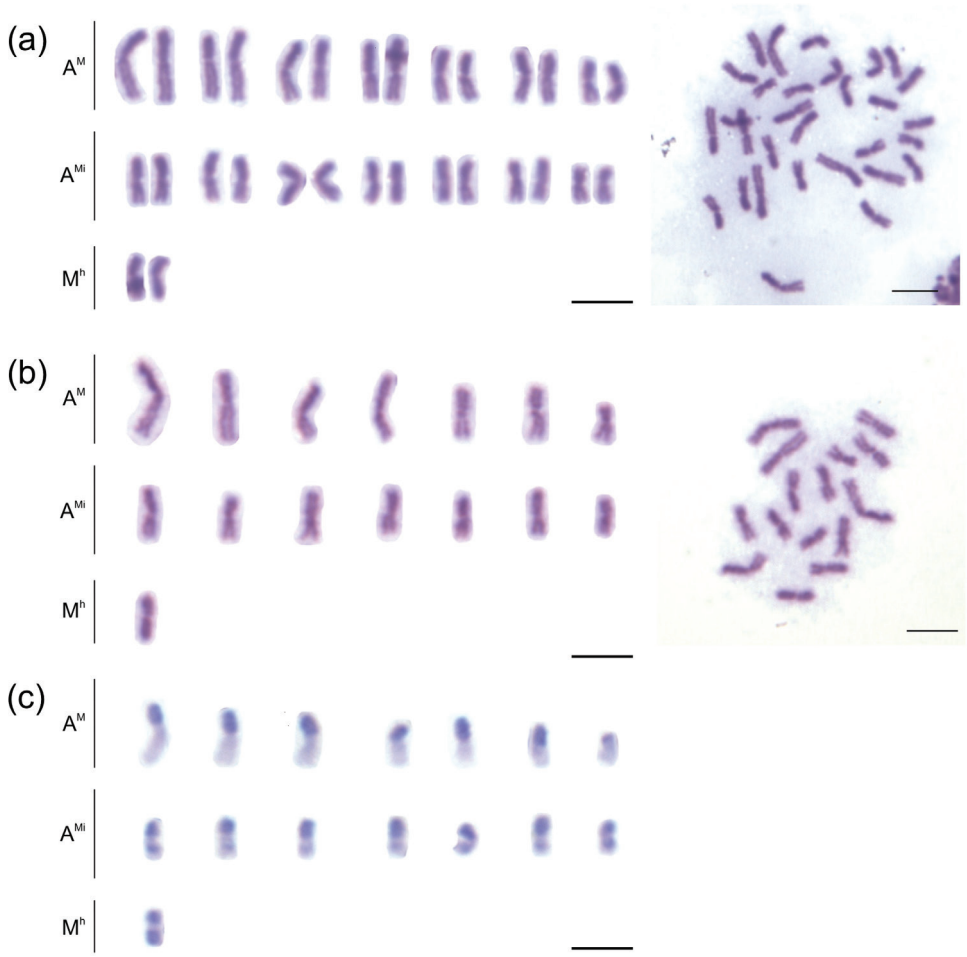

Figure 1. Mitotic karyotypes of *Melitoma segmentaria*. **a** Giemsa staining (female) **b–c** C-banding (male). Bar=5μm.

tern and the nomenclature proposed by Imai (1991), the chromosomes can be classified into three different types: seven pseudo-acrocentric pairs (A^M) with one heterochromatic arm, seven pseudo-acrocentric pairs with an interstitial heterochromatin (A^{Mi}), and one totally heterochromatic metacentric pair (M^h) (see Fig. 1c). According to Imai (1991), pseudo-acrocentric chromosomes are the result of a centric fission, followed by a significant addition of heterochromatin in the telomere region, in order to restore the stability of the chromosome. The entirely heterochromatic metacentric pair may arise from the centric fusion of two heterochromatic acrocentric chromosomes (A^h). A fully heterochromatic metacentric chromosome is uncommon, and this morphological type is found in some supernumerary and Y-chromosomes (Imai 1991, Costa el al. 1992, Camacho et al. 2000, Lopes et al. 2008). All individuals analyzed, both females and males, possess this entirely heterochromatic chromosome, which in-

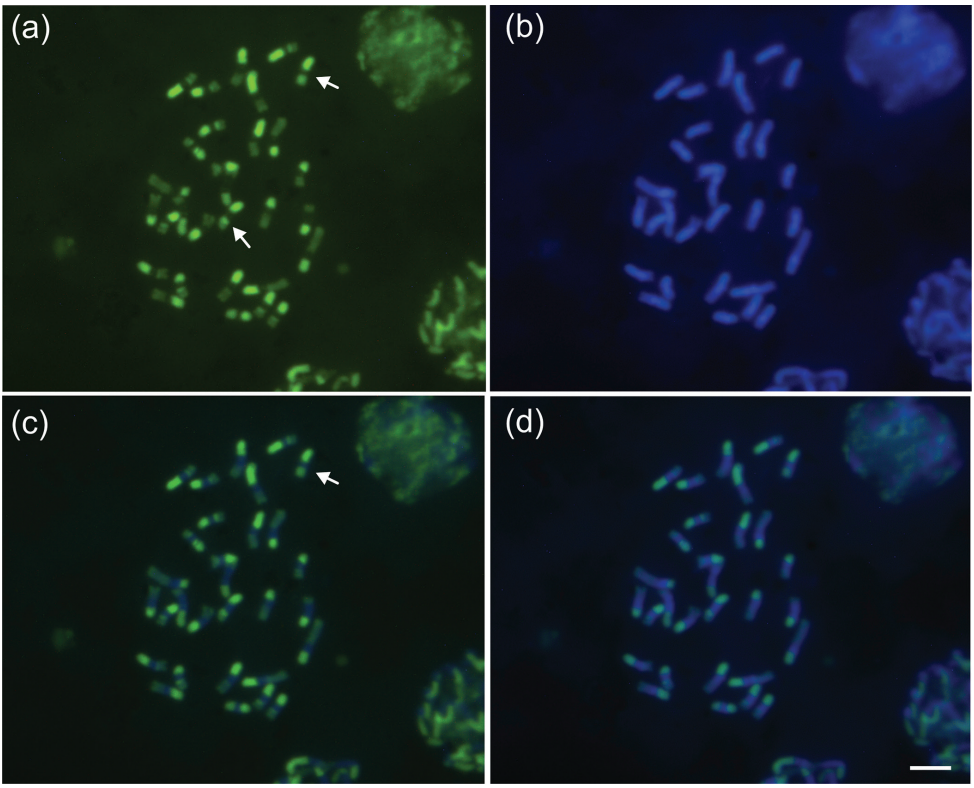

Figure 2. Female mitotic karyotypes of *M. segmentaria* stained with fluorochromes: **a** CMA_3 **b** DAPI **c** CMA_3/DAPI and **d** DAPI/CMA_3. Arrows indicate entirely heterochromatic metacentric chromosomes (M^h). Bar=5μm.

dicates that it is a part of the autosome complement, and hence, it has not been treated as a supernumerary chromosome.

The pattern of heterochromatin distribution in *M. segmentaria* is similar to that observed in most of the studied Meliponini species (Rocha et al. 2003, Carvalho and Costa 2011, Miranda et al. 2013), where most of the chromosomes in the complement have a single heterochromatic arm. This seems to agree with the "minimum interaction hypothesis," proposed by Imai et al. (1988), as the main mechanism of karyotype evolution in these bees. According to this hypothesis, one metacentric chromosome breaks apart at the centromere producing two acrocentric chromosomes. Therefore, due to the instability of these acrocentric chromosomes, the repetitive DNA starts an *in-tandem* growth at the telomere region, leading to chromosomes with a heterochromatic arm (see Imai et al. 1988), as observed here in *M. segmentaria*. However, this pattern is very different from that observed in the solitary bee *Euglossa carolina* (Linnaeus, 1758) (Fernandes et al. 2013), suggesting that alternative mechanisms of karyotype change may occur through the evolutionary diversification of these species. More detailed karyotype studies are needed to point out the trend in the karyotype evolution of solitary bees.

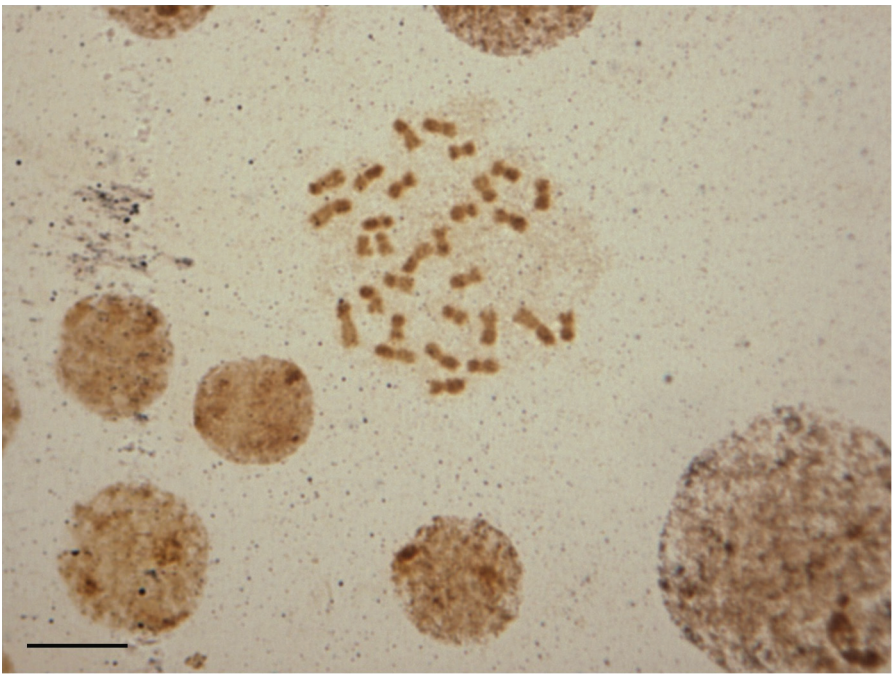

Figure 3. Female mitotic chromosomes of *M. segmentaria* submitted to silver-nitrate staining. Dark regions on the heterochromatin arms indicate silver staining. Bar=5μm.

Chromosome staining with the fluorochromes CMA_3 and DAPI (Fig. 2) shows that heterochromatin has an apparently homogeneous constitution. However, the fluorochrome CMA_3 shows that the heterochromatin present in the chromosomal arms of *M. segmentaria* is more GC-rich than AT-rich. DAPI in *M. segmentaria* marked the centromeric and pericentromeric regions of the chromosomes, indicating that these regions are rich in AT base pairs. In Meliponini bees the heterochromatin is rich in AT base pairs (it is therefore $DAPI^+$) (Brito et al. 2003, Rocha et al. 2003, Lopes et al. 2008). The karyotype of *M. segmentaria* shows heterochromatin richness that is different from the eusocial bees. A similar result has also been observed by Fernandes et al. (2013) in the bee *E. carolina*. Taken together, these results suggest that the evolution of repetitive DNA, the main component of heterochromatin, evolves in different ways in social and solitary bees. However, this conclusion must be treated with caution, because data on only two solitary bees are available and this needs further evaluation.

In order to identify the position of the NORs in the genome of *M. segmentaria*, impregnation with silver nitrate was performed (Fig. 3). However, the methodology used was not efficient enough to indicate the location of the NOR. A particular pattern found in the chromosomes of *M. segmentaria* was a result of silver staining of the heterochromatic chromosomal arms. Overall, NORs were associated with the GC-rich regions, as observed in the bee genus *Friesella* Moure, 1946 (Mam-

pumbu and Pompolo 2000), *Partamona* Schwarz, 1939 (Brito-Ribon et al. 2005, Martins et al. 2013), and *Melipona* Illiger, 1806 (Maffei et al. 2001). The relationship between the NORs and the CG-rich regions was also suggested for the other Hymenoptera species (Cardoso et al. 2012). The recurrent relationship between CMA_3^+ and Ag-NOR staining was also observed in the present study, but the positive Ag-NOR staining in the heterochromatic regions of the diploid chromosome set, was unlikely to indicate the actual position of the NOR. Multiple positive Ag-NOR staining, coincident with C-banding and CMA_3^+ staining, was reported for the stingless bee *Scaptotrigona xanthotricha* (Duarte et al. 2009), and now here for *M. segmentaria.*

The silver impregnation technique located the NOR by staining the proteins present in this region. Sumner (1990) reported that this method was used to visualize heterochromatic regions that were not associated with NORs in various organisms. Therefore, this suggested that the proteins associated with the NORs were qualitatively similar to those encountered in the heterochromatic blocks of *M. segmentaria*. Our results raised issues about the entire effectiveness of Ag-NOR staining, to correctly identify the NOR in all taxa. Future studies, using specific probes for NORs, by means of the fluorescence *in situ* hybridization (FISH) technique, might help to elucidate this.

This study is the first detailed karyotype characterization of the *Melitoma* species, bringing to light several chromosome features, such as, chromosome number, morphology, heterochromatin pattern, and base pair richness. Characterizations of the karyotype of other species of solitary bees and of the genus *Melitoma*, coupled with the use of banding and staining techniques are needed, to obtain a better understanding of the chromosomal evolution in Apidae.

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RESEARCH ARTICLE

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Molecular cytogenetic analysis of the crucian carp, *Carassius carassius* (Linnaeus, 1758) (Teleostei, Cyprinidae), using chromosome staining and fluorescence *in situ* hybridisation with rDNA probes

Aneta Spoz[1], Alicja Boron[1], Katarzyna Porycka[1], Monika Karolewska[1], Daisuke Ito[2], Syuiti Abe[3], Lech Kirtiklis[1], Dorota Juchno[1]

1 *Department of Zoology, Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn, M. Oczapowskiego Str. 5, 10-718 Olsztyn, Poland* **2** *Instituto Gulbenkian de Ciência, Rua da Quinta Grade, 6, 2780-156, Oeiras, Portugal* **3** *Sanriku Fisheries Research Center, Department of Revitalization for Sanriku-region, Iwate University, 3-75-1, Heita, Kamaishi 026-0001, Iwate, Japan*

Corresponding author: *Alicja Boron* (alibo@uwm.edu.pl)

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Abstract

The crucian carp *Carassius carassius* (Linnaeus, 1758) is a species with restricted and decreasing distribution in Europe. Six males and six females of the species from the Baltic Sea basin in Poland were examined to show sequentially CMA_3/$AgNO_3$ staining pattern, DAPI staining, and, for the first time in literature, molecular cytogenetic analysis using double-colour fluorescence *in situ* hybridisation (FISH) with 28S and 5S rDNA probes. The karyotype consisted of 20 m, 36 sm and 44 sta chromosomes, NF=156. The $AgNO_3$ stained NORs were most frequently located terminally in the short arms of two sm and two sta elements, and CMA_3-positive sites were also observed suggesting abundant GC-rich repetitive DNA in the regions. Other CMA_3-positive sites in the short arms of six to ten sm and sta chromosomes were detected. The results based on 28S rDNA FISH confirmed the location of rDNA sites. DAPI-negative staining of NORs suggested the scarcity of AT-rich DNA in the regions. FISH with 5S rDNA probe revealed 8–14 loci (ten and 12 in respectively 49 and 29% of metaphases). They were located in two sm and eight to ten sta chromosomes and six of them were larger than others. Simultaneously, mapping of the two rDNA

families on the chromosomes of *C. carassius* revealed that both 28S and 5S rDNA probes were located in different chromosomes. Molecular cytogenetic data of *C. carassius* presented here for the first time give an important insight into the structure of chromosomes of this polyploid and declining species and may be useful in its systematics.

Keywords
Cyprinidae, CMA_3, FISH with rDNA, molecular cytogenetics, NOR-phenotype, polyploid species

Introduction

The genus *Carassius* Jarocki, 1882 is a fish group of polyploid origin as are some other cyprinids of subfamilies Cyprininae and Barbinae s.l., e.g. *Cyprinus* Linnaeus, 1758 and *Barbus* Cuvier, 1816 (Vasil'ev 1985, Le Comber and Smith 2004). The importance of polyploidy in the evolution of Teleostei fishes is evident, as they are known for their advantage to survive in different environmental conditions (Gui and Zhou 2010, Yuan et al. 2010). Polyploid species are a useful model system for comparative investigations of the evolutionary process accompanied by polyploidisation at genome and chromosome level (Yuan et al. 2010, Mani et al. 2011, Pereira et al. 2012, Kumar et al. 2013, Li et al. 2014).

The crucian carp, *Carassius carassius* (Linnaeus, 1758), native to Europe, is widely distributed from the northern France to the Danube drainage and Siberia, and from England in the north to the Alps in the south. This species is adapted to both a wide range of temperature and low oxygen content and prefers densely vegetated water bodies–backwaters and oxbows of lowland rivers, and lakes (Szczerbowski and Szczerbowski 2002, Freyhof and Kottelat 2008).

The crucian carp is included in the least concern IUCN category but is regarded as disappearing in many water bodies of its range (Freyhof and Kottelat 2008). The area of distribution of this species in Poland decreased during the last two decades (Witkowski and Grabowska 2012). In recent years, interspecific hybrids have been frequently recorded between the crucian carp and the introduced Prussian carp *C. gibelio* (Bloch, 1782), the goldfish *C. auratus* (Linnaeus, 1758) and the common carp *Cyprinus carpio* Linnaeus, 1758 (Sayer et al. 2011, Wouters et al. 2012, Mezhzherin et al. 2012, Rylková et al. 2013). Hybridisation threats to the conservation of this species may lead to displacement of the genome of *C. carassius* by genomes of hybrids. In context of the genetic conservation of this species, it is important to determine its taxonomic diagnostic features possibly at all levels of its organisation including the chromosomal level.

The karyotype of this species has been described by Makino (1941), Chiarelli et al. (1969), Kobayasi et al. (1970), Hafez et al. (1978), Sofradžija et al. (1978), Raicu et al. (1981), Vasil'ev (1985), Vasil'ev and Vasil'eva (1985), Kasama and Kobayasi (1991) and Wang et al. (1995). For a long time there had been only two reports on the chromosomal distribution of the NORs (Mayr et al. 1986, Takai and Ojima 1986), but

data involving the karyotype and some of conventional chromosome banding pattern were recently published by Knytl et al. (2013a, b).

The location of ribosomal genes in the chromosomes is commonly used as very informative cytogenetic features (Zhu et al. 2006, Zhu and Gui 2007, Singh et al. 2009; Mani et al. 2011, Pereira et al. 2012, Kumar et al. 2013). In higher eukaryotes, ribosomal RNA genes (rDNAs) are organised into the nucleolus forming major rDNA (45S) family composed of clusters of multiple copies of tandem repeated units with coding regions for 18S, 5.8S and 28S rRNA genes and non-nucleolus forming minor rDNA (5S) family (Pendas et al. 1993).

In the present study, the crucian carp *C. carassius* was examined for the chromosomal distribution of the nucleolar organiser regions (NORs) using sequential staining with silver nitrate ($AgNO_3$), chromomycin A_3 (CMA_3), and DAPI staining. Moreover, fluorescence *in situ* hybridisation (FISH) with 28S (major) and 5S (minor) rDNA probes was performed. This is the first report of simultaneous localisation of two rDNA families (45S and 5S rDNA) in chromosomes of *C. carassius*. The ribosomal gene distribution data extend our knowledge on the cytotaxonomy and gave us information about functional structure of the chromosomes in this polyploid and declining species.

Material and methods

Fish specimens

In total 12 individuals, six males and six females of *C. carassius* (of the average length and body weight respectively, 165.0 mm and 140.0 g for females, and 151.0 mm and 124.0 g for males) were studied. They were collected from the Kortowskie Lake (53°45'43"N; 20°26'42"E), the Pregola River drainage (Baltic Sea basin) by net and then transported alive to the laboratory. Species identification followed Szczerbowski and Szczerbowski (2002) and Freyhof and Kottelat (2008). As typical for *C. carassius*, the specimens examined had a light (non-pigmented) peritoneum, the external morphology (deep body, rounded dorsal fin, small serration on the last unbranched ray in the dorsal fin) and general colouration (golden colour of the dorsal and lateral parts of the body).

Chromosome preparation and staining

Mitotic chromosome preparations were made from each individual following Boron et al. (2011). First, live fish were injected with a dose of 1ml of 0.05% colchicine solution per 100g body weight. The experiments followed ethical conducts, and fish were anaesthetized using MS 222 prior to sacrificing. Mitotic chromosomes were obtained from kidney cell suspensions using the air-drying method. The kidney cells were exposed to a hypotonic solution (0.075M KCl) for 30 min and fixed in methanol : acetic acid (3:1).

Chromosomes were stained with a solution of 4% Giemsa (pH=6.8) and then classified according to Levan et al. (1964). Meta- (m) and submetacentric (sm) chromosomes were classified as biarmed, whereas subtelo- and acrocentric (sta) as uniarmed elements. Chromosomes were counted in at least 20 metaphase plates in each individual and were analysed using MultiScan software with the additional Karyotype supplement.

Chromosome slides of three males and three females were sequentially stained with $AgNO_3$ and CMA_3 according to Sola et al. (1992). The active AgNOR sites and CMA_3-positive sites were counted in 15 metaphase plates from each individual, using MultiScan software with the additional Karyotype supplement.

Probes and fluorescence *in situ* hybridisation (FISH)

Single colour FISH with human 28S rDNA probe or double-colour FISH with loach 5S and human 28S rDNA probes were used according to Fujiwara et al. (1998) and Boron et al. (2009). The 5S rDNA probe was labelled with biotin-16-dUTP using Biotin-Nick Translation Mix kit (Roche), while the 28S rDNA probes were labelled with digoxigenin-11-dUTP using the DIG-Nick Translation Mix kit (Roche), according to the manufacturer's instructions. The chromosome slides were initially incubated with RNase for 60 min at 37 °C in a moist chamber. After denaturation for 1 min in 70% formamide (FA)/2×SSC, chromosome slides were dehydrated in an ethanol series, 70% for 5 min and 80%, 90%, and 100% for 2 min, each at 20 °C. Hybridisation with a mixture containing denatured rDNA probes, Bovine Serum Albumin, 50% dextran sulphate, 20×SSC, and double-deionised water was performed at 37 °C in a moist chamber. Post-hybridisation washes were performed in 50%FA/2×SSC at 37 °C for 20 min, 2×SSC and 1×SSC for 20 min each, and 4×SSC for 5 min. 5S and 28S rDNA probes were detected with Avidin-Fluorescein (Roche) and Anti- Digoxigenin-Rhodamine (Roche), respectively. Then, chromosomes were counterstained with DAPI in Antifade solution (Vector Laboratories). We show here both single colour and dual colour FISH with rDNAs because firstly prepared single colour FISH revealed DAPI banding pattern. This pattern turned out to invisible after dual colour FISH.

Hybridisation signals in at least 15 metaphase plates of each individual were observed under a Nikon Eclipse E800 fluorescence microscope using a Nikon B-2A filter for a single colour FISH and black and white CCD camera Pixera Penguin 150CL-CU (Pixera), and a Nikon Eclipse 90i fluorescence microscope equipped with ProgRes MFcool camera (Jenoptic) for capturing the images of a dual colour FISH. The images were processed using Penguin Mate ver. 1.0.8. software for RGB pseudocolour imaging (Pixera) and Lucia ver. 2.0 (Laboratory Imaging).

Voucher specimens were preserved frozen and deposited at the Department of Zoology, University of Warmia and Mazury in Olsztyn, Poland.

Results

Karyotype and banding patterns

The crucian carp from the Kortowskie Lake exhibits a diploid chromosome number of 100 (Fig. 1a) without any supernumerary chromosomes in 369 (94.4%) out of 391 analysed metaphase plates. The karyotype consisted of 20 m, 36 sm and 44 sta chromosomes (Fig. 1b). The chromosome arm number (NF) was counted as 156. The first submetacentric pair (11th pair) was easily recognisable in all metaphase plates, being the largest elements in the chromosome complement. No variability in the chromosome formula was observed and heteromorphic sex chromosomes were not detected.

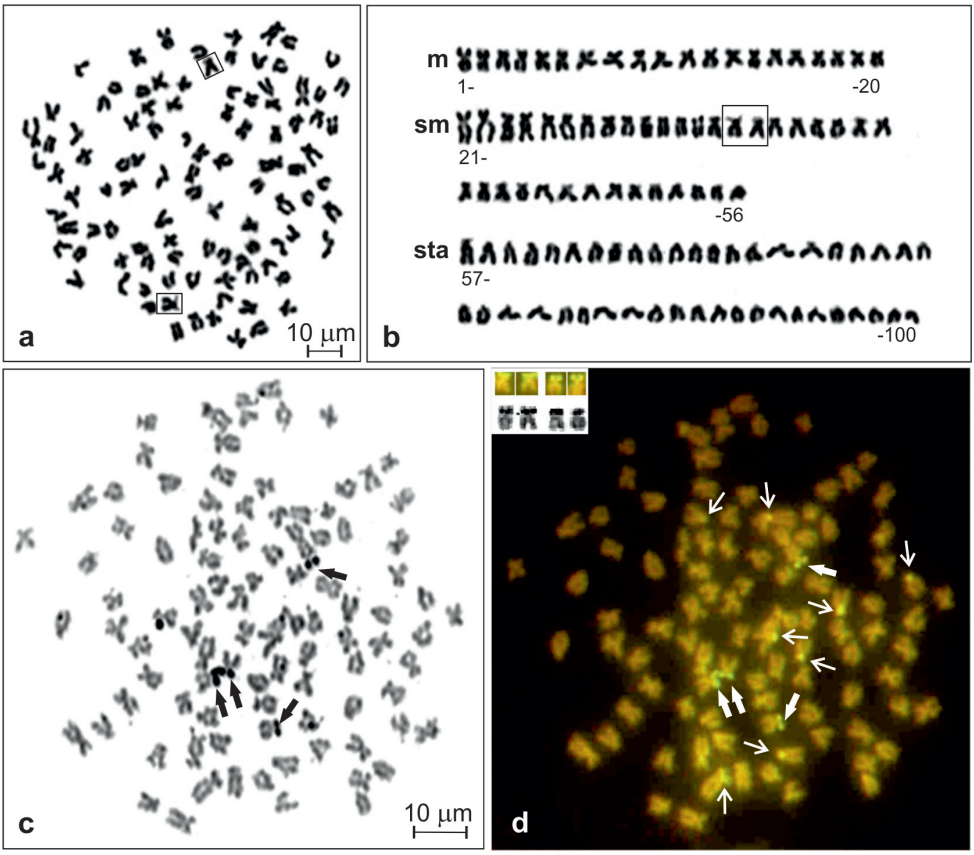

Figure 1. Giemsa stained metaphase (**a**), corresponding karyotype of *C. carassius* (**b**), and metaphase spread sequentially stained with $AgNO_3$ (**c**) and CMA3 (**d**). NOR chromosomes shown in frames (in **a** and **b**), Ag-NORs and corresponding CMA3-positive sites shown by thick arrows (in **c** and **d**) and shown in inset (in **d**), other CMA3-positive sites shown by thin arrows (in **d**).

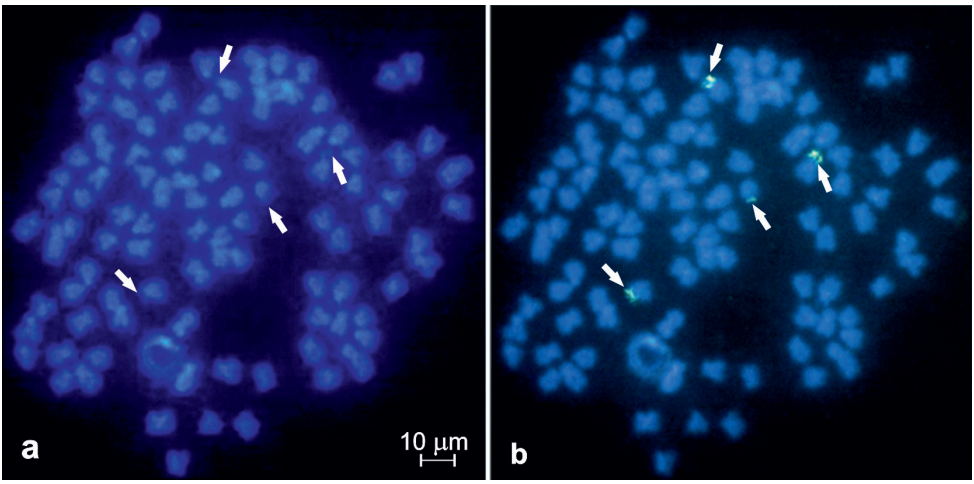

Figure 2. Metaphase plate of *C. carassius* DAPI stained (**a**) and with a single colour FISH (**b**) with 28S rDNA probe. 28S rDNA hybridisation signals shown by arrows.

$AgNO_3$ stained active nucleolus organiser regions (AgNORs) were located terminally at the short arms of two sm and two st chromosomes (Fig. 1c). After sequential staining with CMA_3, all signals were observed as a distinct bright fluorescence, suggesting abundant GC-rich repetitive DNA sequences in the regions (Fig. 1d). Among 90 metaphases of six individuals, two to four CMA_3-positive sites corresponding to AgNORs were detected, but 57.8% of metaphases showed four bright signals. Three and two such sites were observed, respectively, in 31.1 and 11.1% of analysed metaphases. In addition, there were extra CMA_3-positive sites located at the short arms of six to ten sm and sta chromosomes. Most frequently (in 52.2% of metaphases) eight such sites at four of each of sm and sta elements (Fig. 1d) or six (in 32.2% of metaphases) sites at three of each of sm and sta were observed.

One of the submetacentric chromosomes (chromosome no. 35 of pair 18, shown in frame in Fig. 1a–b), possessing clearly visible secondary structure along its short arm, was easily distinguishable among others in all metaphase plates stained with Giemsa.

DAPI-counterstained chromosomes have shown some slightly visible AT-rich pericentromeric heterochromatic regions of 12–14 sta and at the short arms of four to six sm (Fig. 2a–b). However, they were not detected in the metaphase plates after using dual colour FISH that such the chromosomal regions were dimly DAPI-stained (Fig. 3a).

FISH mapping of 28S rDNA loci

Single FISH using 28S rDNA probe analysed in 68 metaphase plates of two females and two males and dual colour FISH analysed in 243 metaphase plates of four females and four males revealed either three or four loci in their chromosome complement. In most of the metaphase plates (76.2%), the signals were found in the short arms of two

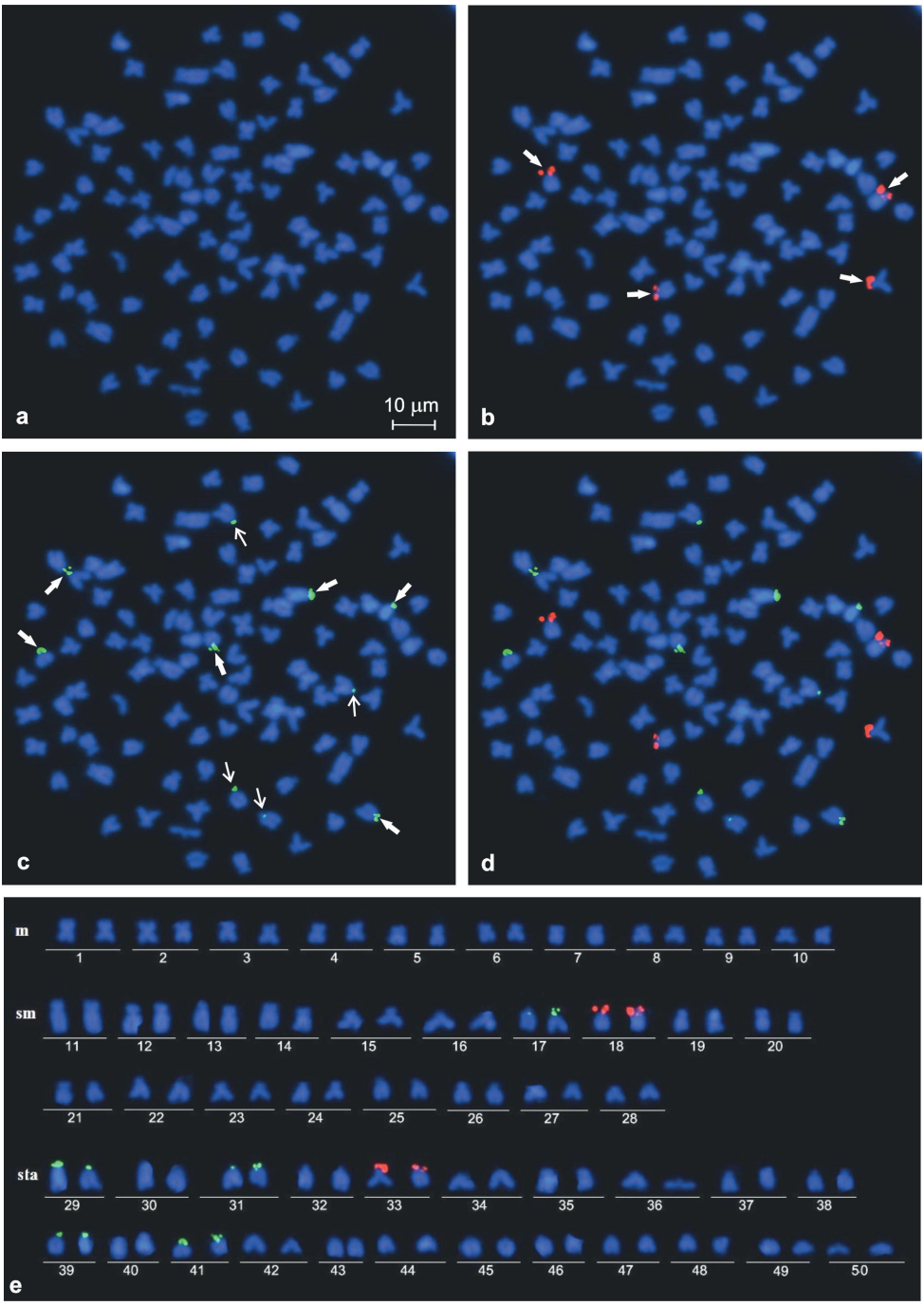

Figure 3. Representative mitotic metaphase plates (**a–d**) and corresponding karyotype of *C. carassius* (**e**): **a** DAPI stained and **b–d** most frequent hybridisation pattern after dual colour FISH with four 28S rDNA sites (**b**), ten 5S rDNA sites (**c**) and both rDNA probes (**d**). Six stronger and four weaker 5S rDNA hybridisation sites (**c**) shown by thick and thin arrows, respectively.

each of sm and st chromosomes (Figs 2b, 3b). Three hybridisation sites were observed commonly as intense and large signals, whereas the signal in the fourth site was smaller and weaker than the other three sites. DAPI-negative staining of the observed NORs suggested the scarcity of AT-rich DNA in the regions (Figs 2a, b, 3a, b). In the rest of the analysed metaphase plates (23.8%), three 28S rDNA sites were observed in the short arms of two sm and one sta elements. Numerous metaphases showed close association of NORs involving two or sometimes three chromosomes.

FISH mapping of 5S rDNA loci

FISH with 5S rDNA probe analysed in 243 metaphase plates of four males and four females revealed an unexpectedly large number of loci, from eight to 14. The obtained hybridisation signals had different intensities on various chromosomes and could be classified as strong and weak (Fig. 3c–d). All individuals frequently showed 10 (Fig. 1c) or 12 such loci in respectively 48.6% and 29.2% of metaphase plates. They were located at the short arms of two sms (pair 17 in Fig. 3e) and at the short arms or in a subcentromeric position of eight to ten sta chromosomes (pairs 29, 31, 39 and 41 in Fig. 3e). Six hybridisation sites of 5S rDNA were stronger than the other four to six (Fig. 3c–e). Among 15.2% and 7.0% of the rest of metaphase plates, the 5S rDNA loci were located, respectively, in eight and 14 chromosomes. Usually, in metaphase plates containing 14 signals, two signals were very weak.

Thus, *C. carassius* was characterised by the modal number of ten 5S rDNA loci. Signal heteromorphism was detected on the homologous chromosome of pairs 17 and 31 (Fig. 3e). Both classes of rDNA probes were always located in different chromosomes and co-localisation in the same chromosome was not observed (Fig. 3d).

No sex-dependent variability in the cytogenetic features was found.

Discussion

Undoubtedly, the crucian carp *C. carassius* possesses 2n=100 chromosomes in its somatic cells but data on the karyotype reported in literature somewhat differ (Table 1). The reason for this could be that the karyotype of the crucian carp contains a lot of very small chromosomes which are similar in size. The problem mainly concerns discrimination between sm and sta chromosomes as it occurs in the karyotype of a related species *C. gibelio* (Boroń et al. 2011). The karyotype obtained in the present study with a larger number (56) of biarmed than (44) of uniarmed chromosomes is the same as that supposed by Knytl et al. (2013a, b). Similar karyotype characterised by the largest sm pair was described by Kobayasi et al. (1970), Sofradžija et al. (1978), Hafez et al. (1978), and Kasama and Kobayasi (1991) in *C. carassius*.

The crucian carp and other *Carassius* species distributed in Europe were recognised as monophyletic lineages (Rylková et al. 2013). Among them, only *C. carassius*

Table 1. Cytogenetical data of the crucian carp, *C. carassius*. Symbols of chromosomes: m – metacentric, sm – submetacentric, sta – subtelo- to acrocentric, NF – number of chromosome arms.

L.p	Locality	2n	Karyotype	NF	Cytogenetic features	Reference
1.	-	94	-	-	-	Makino 1941
2.	-	104	20m+72sm+12a	196	-	Chiarelli et al. 1969
3.	the Netherlands (Baltic basin)	100	20m+40sm+40a	160	-	Kobayasi et al. 1970
4.	France (Garonne drainage)	100	20m+44sm+36a	164	-	Hafez et al. 1978
5.	Drina R. (Danube), Bosnia	100	52m, sm+48sta	152	-	Sofradžija et al. 1978
6.	Danube R., Romania	50	20m+12sm+18sta	82	-	Raicu et al. 1981
7.	Water bodies in Moscow region, Russia (Volga drainage)	100	48m, sm+52sta	148	-	Vasil'ev 1985, Vasil'ev and Vasil'eva 1985
8.	Elbe R., Czech Republic	100	-	-	AgNOR	Mayr et al. 1986
9.	the Netherlands (Baltic basin)	100	20m+40sm +40a	160	-	Kasama and Kobayasi 1991
10.	Tarim R., Xinjiang, China	100	32m+34sm+34sta	166	-	Wang et al. 1995
11.	Elbe R., Czech Republic	100	20m+36sm+44sta	156	C bands, AgNOR, DAPI/CMA_3	Knytl et al. 2013a,b
12.	Kortowskie Lake, Pregola R. drainage, Poland	100	20m+36sm+44sta	156	AgNOR/CMA_3, 45S and 5S rDNA (FISH)	present study

is characterised by 2n=100 chromosomes and can be easily identified morphologically (Szczerbowski and Szczerbowski 2002). Identification of other species according to the chromosome number is complicated by the occurrence of both diploid and triploid specimens within *C. gibelio*, *C. langsdorfii* Temminck & Schlegel, 1846 and *C. auratus* (Linnaeus, 1758) (Rylková et al. 2013).

Most of the cyprinid species, for example those from the subfamilies Leuciscinae, Gobioninae and Danioninae, are characterised by 2n=50 or 2n=48 chromosomes (e.g. Vasil'ev 1985, Rab and Collares-Pereira 1995). The polyploids exist in the subfamily Cyprininae s.l., within the following tribes recognised by Yang et al. (2010): cyprinins (e.g. *Cyprinus* and *Carassius*), barbins (e.g. *Barbus* and *Tor* Gray, 1834) and oreinins (e.g. *Schizothorax* Heckel, 1838). The karyotype of *C. carassius* is similar to other polyploid cyprinin species (Le Comber and Smith 2004, Singh et al. 2009, Mani et al. 2011) possessing 100 chromosomes may be shown as 2n=4X=100. We assume, following Vasil'ev (1985) and Buth et al. (1991), that the haploid number of chromosomes equals n=25, so, they are tetraploids. It would be expected that the chromosomes in these species formed tetravalents during the prophase of meiosis I. Occurrence of only bivalents indicates the 'diploid nature' of *C. carassius*. So, this species as some others of the genera mentioned above have evolved via formation of polyploids and subsequent diploidisation process (Vasil'ev 1985, Buth et al. 1991).

The number of four AgNORs (two sm and two st) characterises the karyotype of *C. carassius* (Knytl et al. 2013b), but they varied from two to four as was shown in this study consistently with their transcriptional activity during the preceding interphase. Intraspecific and intraindividual variation of AgNORs results from that Ag-staining solely detects the products of active 18S, 5.8S and 28S rDNA expression in the preceding interphase (Reeder 1990).

We documented that the AgNOR sites were CMA_3 positive similar to what is found in many other Teleostei (Knytl et al. 2013b). It can be interpreted as a high copy number of repeating units of rDNA (Gromicho and Collares-Pereira 2007). The obtained results support the hypothesis that CMA_3 staining of GC-rich heterochromatin shows all active and non-active NORs in the chromosomes. However, the only four of numerous GC-rich DNA heterochromatin sites in the karyotype of *C. carassius* were associated with major ribosomal sites. The CMA_3-positive sites being NOR-negative may be related to nucleolar dominance phenomenon reported in other organisms and other taxa of Teleostei, and in some hybrids and species of hybrid origin (Gromicho et al. 2005). The additional CMA_3-positive sites were not found in *C. carassius* by Knytl et al. (2013b).

The karyotype of *C. carassius* after DAPI staining described by Knytl et al. (2013b) was uniform. We gained slightly visible AT-rich heterochromatic regions of DAPI-counterstained chromosomes in single colour FISH staining, whereas the chromosome DAPI differentiation was not revealed using dual colour FISH. The differences may result from the level of chromatin condensation and/or technical reasons. DAPI-negative staining of the NORs reported here and described in, for example, *Rhodeus amarus* (Bloch, 1782) (Kirtiklis et al. 2014) reflected the occurrence of GC-rich heterochromatin and the scarcity of AT-rich DNA in these regions.

The results from FISH with 28S rDNA confirmed for the first time in literature that the karyotype of *C. carassius* (2n=100) is characterised by the conservative number of NORs – four – located in the short arms of two sm and two st chromosomes. It was mentioned by Knytl et al. (2013b) that this NOR chromosomal pattern supported a hypothesis of the palaeotetraploidy of the crucian carp genome as was earlier suggested by Vasil'ev and Vasil'eva (1985). Similarly, five located NORs were found in the karyotype of a related species *C. gibelio* with 162 chromosomes (Zhu and Gui 2007). According to Foster and Bridger (2005), the terminal position of 45S rDNA, considered as a primitive stage in Teleostei, would promote chromosomal dispersion due to their proximity within an interphase nucleus. The presence of a single chromosomal pair bearing 28S rDNA was assumed to represent an ancestral condition in fishes, since this pattern had been reported in species representing all so far investigated fish orders (Martins and Wasko 2004, Nakajima et al. 2012). Taking this into consideration the presence of two pairs of NORs found in *C. carassius* may be connected with the polyploid origin of the species. A similar pattern with two or more pairs of NOR chromosomes is known in species from the genus *Tor* (2n=100) (Singh et al. 2009, Mani et al. 2011). However, two or multiple NORs were observed in many other non-polyploid cyprinid species with 2n=50 chromosomes (Pereira et al. 2012, Kumar et al. 2013).

The weak or missing signal of hybridisation in one out of the four NORs in the karyotype of *C. carassius* could be due to either a low copy number of 28S rDNA or a deletion of these genes, or due to technical reasons (Pendas et al. 1993, Fujiwara et al. 1998). Alternatively, it could be an effect of chromosomal rearrangement associated with the occurrence of transposable elements (Pearson et al. 2005). Rapid chromosome rearrangement was proposed as exiting in the postpolyploidy genome of *C. gibelio* according to size variation and 45S rDNA distribution (Zhu and Gui 2007).

The FISH localisation of the 5S rDNA revealed that these sequences are spread in at least eight chromosomes. *Carassius auratus* (2n=100) is characterised by 5S rDNA large hybridisation sites located at the short arms of two st and from two to eight smaller 5S rDNA sites whereas a triploid form of *C. gibelio* (3n=162) had three larger sites and from six to 18 small ones (Zhu et al. 2006). Strong signals of 5S rDNA at the short arms of two to four pairs of acrocentric or subtelocentric and several additional weak signals were also observed in the karyotype of *Cyprinus carpio* (Inafuku et al. 2001). Multiple loci for the 5S ribosomal sequences and their varying hybridisation signals seem to be typical for *Carassius* and *Cyprinus* species. However, the location of 5S rDNA sites in the karyotypes of *C. carassius*, *C. auratus* and *C. gibelio* does not confirm the opinion about conservative pattern of 5S rDNA loci distribution in closely related species (Gromicho et al. 2006, Singh et al. 2009, Mani et al. 2011).

Commonly in Teleostei, there is a single locus for the 5S ribosomal sequences, which is regarded as an ancestral condition while the hybridisation pattern with two or more loci may be considered as a derived state (Martins and Wasko 2004, Singh et al. 2009, Nakajima et al. 2012, Kumar et al. 2013). Apart from the above mentioned species, two and more loci of 5S rDNA were found also in some natural hybrids and/or polyploid taxa (Martins and Wasko 2004, Gromicho et al. 2006, Mani et al. 2011, Pereira et al. 2012) as well as in some diploid species (Kirtiklis et al. 2010). This requires verification whether the numerous chromosomes containing the sequence of 5S rDNA are an idenfining marker of species which are generally considered as diploids but, from evolutionary point of view, being actually diploidised polyploids (after polyploidisation event).

The 5S rDNA clusters in fishes seem to be most frequently located at interstitial chromosome sites as they were found in most fish species in different orders (Martins and Wasko 2004). A non-terminal location of this rDNAs could reflect an ancestral condition of the chromosomal organisation (Martins and Wasko 2004, Nakajima et al. 2012). The 5S rDNA loci observed in the karyotype of *C. carassius* (Fig. 1e) near the centromere region and in a subcentromeric position as well as similarly located such loci in the karyotypes of *C. gibelio* and *C. auratus* (Zhu et al. 2006) may reflect chromosomal rearrangements. The activities of repetitive sequences as well as transposable elements are often correlated with genomic sequence elimination and chromosome rearrangements (Zhu et al. 2006). However, additional discrete signals after FISH with 5S rDNA probe may appear as a result of hybridisation to the chromosome regions consisting of repetitive sequences similar to the 5S rDNA fragments (Ferreira et al. 2007).

In most of the described fish species including cyprinids, the two rDNA families are located at different chromosomes (Fujiwara et al. 1998, Singh et al. 2009, Nakajima et al. 2012; Kumar 2013). However, in others, including some cyprinids, the minor rDNA loci are co-localised with the major rDNA loci in the same chromosome (Inafuku et al. 2000, Gromicho and Collares-Pereira 2007, Pereira et al. 2012). In *C. carassius* as well as in *C. gibelio*, the minor and major rDNA clusters are located in different chromosomes (Zhu et al. 2006). This 5S rDNA pattern, with none of the numerous signals overlapping with the 28S, represents a characteristic cytogenetic feature of *Carassius* species.

Conclusion

We updated the information on the karyotype, showed for the first time sequentially $CMA_3/AgNO_3$ banding pattern and also provided new molecular cytogenetic data on the crucian carp *C. carassius* using double-colour FISH with 5S and 28S rDNA probes. The obtained results improve our knowledge about the chromosome structure and physical location of major and minor ribosomal sequences in this fish species. Moreover, the results herein gave an important insight into the molecular cytotaxonomy of the crucian carp a polyploid and declining species and may be useful in its systematics.

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