Cytogenetic and molecular markers reveal the complexity of the genus *Piabina* Reinhardt, 1867 (Characiformes: Characidae)

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Cytogenetic and molecular analyses were carried out in fish representative of the genus *Piabina*. This study specifically involved the species *P. argentea* and *P. anhembi* collected from areas of the Paranapanema and Tietê River basins, Brazil. Our findings suggest that fish classified as *Piabina argentea* in the Paranapanema and Tietê Rivers may represent more than one species. The samples analyzed differed by cytogenetic particularities and molecular analyses using partial sequences of the genes COI and CytB as genetic markers revealed three distinct groups of *P. argentea* with genetic distances sufficient to support the conclusion that the three samples analyzed are three distinct taxonomic units.

Foram realizadas análises citogenéticas e moleculares em representantes do gênero *Piabina*. O estudo envolveu especificamente as espécies *P. argentea* e *P. anhembi* coletadas nas áreas das bacias hidrográficas dos rios Paranapanema e Tietê (Brasil). Os dados sugerem que a espécie *P. argentea* coletada nas bacias dos rios Paranapanema e Tietê podem representar mais de uma espécie. As amostras analisadas diferem por particularidades citogenéticas e nas análises moleculares utilizando-se sequências parciais dos genes COI e CytB, revelando três grupos distintos de *P. argentea* com distâncias genéticas suficientes para sustentar a conclusão de que as três amostras analisadas são unidades taxonômicas distintas.

Key words: DNA Barcoding, Freshwater fishes, Fish, mtDNA, 18S rDNA, 5S rDNA.

Introduction

The Neotropical freshwater fish fauna is considered the richest in the world (Schaefer, 1998). According to Reis *et al*. (2003), approximately 6,000 freshwater fish species are estimated to be found in this region, 4,475 of these species are considered valid and approximately 1,550 are recognized but not described.

Cytogenetic fish data are available for 475 species of Characiformes, 318 species of Siluriformes, 48 species of Gymnotiformes and 199 freshwater species belonging to the superorder Ostariophysi (Oliveira *et al*., 2009). According to Almeida-Toledo *et al*. (2000), the chromosome number among the Neotropical fish species studied ranges from 20 in *Pterolebias longipinnis* (Garman, 1895) (Oliveira *et al*., 1988) to 134 in *Corydoras aeneus* (Gill, 1858) (Turner *et al*., 1992). In some groups the number and morphological features of the chromosomes are conserved while in other freshwater fish groups these characteristics show remarkable variation.

Cytogenetic techniques have been used to characterize populations, species, genera and families, and many of them have proved to be efficient markers in identifying intra and inter-specific chromosome variations (Mantovani *et al*., 2000). The development of specific cytogenetic techniques, mainly conventional chromosome banding techniques, has facilitated accurate chromosome identification and permitted a better understanding of the karyotypic structure. These findings have revealed relationships among species and the mechanisms involved in the evolutionary processes that have occurred in this group of organisms (Almeida-Toledo *et al*., 2000). Recently, the studies in chromosome structure were challenged with the introduction of new molecular cytogenetic techniques that make it possible to analyze specific parts of the chromosomes by using microdissection procedures and *in situ* hybridization with localized genomic probes (Pansonato-Alves *et al*., 2011).

The first cytogenetic studies on the species *Piabina argentea* (Reinhardt, 1867) were carried out with specimens from the Mogi-Guaçu River (São Paulo, Brazil). This sample has a 2n=52 chromosomes (26 m/sm + 26 st/a) (Portela *et al*., 1988) with NORs located in multiple sites in the terminal region of the chromosomes. However, Peres *et al*. (2007) also found specimens of *P. argentea* from the São Francisco river basin with a 2n=52 chromosomes (8M +14 SM +16 ST +14 A) and the NORs located in the terminal region of only one chromosome pair.

The use of molecular markers in taxonomy has enabled breakthroughs in this area of research and has enabled the identification of species with the use of genes or specific genomic segments. Following these trends, Hebert *et al.* (2003) proposed a molecular system of identification of species called DNA barcoding, which is based on using a fragment of the Cytochrome Oxidase I (COI) mitochondrial gene. Many studies have shown the efficacy of this method in identifying many fish species (Valdez-Moreno *et al.*, 2009; Ward , 2009; Lara *et al.*, 2010; Pereira *et al.*, 2011). Some of these findings have resulted in the proposal of new species of fish (Ward *et al.*, 2008; Nguyen & Seifert, 2008; Yassin *et al.*, 2008).

Considering the wide distribution of *P. argentea* and the endemic distribution of *P. anhembi* da Silva & Kaefer, 2003, as well as the small number of cytogenetic studies for this group, the aim of this study was to characterize different populations of *P. argentea* and *P. anhembi* using cytogenetic markers (Giemsa, C-banding and Ag-NORs) and molecular-cytogenetic markers (FISH using probes for the ribosomal genes 5S rDNA and 18S rDNA) and thereby contribute new information about the karyotype of these species. Additionally, in order to verify the species status of the widely distributed *P. argentea,* we assessed partial sequences of the COI and Cytochrome B (CytB) mitochondrial genes using the parameters proposed by the DNA barcoding technique. Molecular analysis combined with cytogenetic data should provide information that is relevant to establishing an accurate species status for this fish group.

Material and Methods

We analyzed forty-five specimens of the fish species *P. argentea* (22 males and 23 females) from the Paranapanema river (one local sample) and the Tietê river (two local samples) and 13 specimens of *P. anhembi* (upper Tietê River basin) located in São Paulo State, Brazil (Table 1; Fig. 1). These samples were collected from 2006 to 2008. After the samples were processed for chromosome preparation, the specimens were fixed in 10% formalin, preserved in 70% alcohol, identified and deposited in the fish collection of the Laboratório de Biologia e Genética de Peixes (LBP), UNESP, Botucatu (São Paulo State, Brazil) (collection numbers LBP 6743, 6744, 6745, 6742, and 4622). Small fragments of muscle or fin were collected and preserved in 95% ethanol for molecular analysis.

Cytogenetic analysis

Mitotic chromosome preparations were obtained from kidney and gill fragments following the technique used by Foresti *et al.* (1981). NOR sites were identified by silver nitrate following the technique proposed by Howell & Black (1980), and C-banding was performed following the protocol described by Sumner (1972).

Fluorescent *in situ* hybridization (FISH) was conducted to locate the rDNA sites on chromosomes. The 18S probe and 5S probe were generated following the protocol described by Pinkel *et al.* (1986) with 77% of stringency. The 18S probe was obtained from *Prochilodus argenteus* (Agassiz, 1829) following the protocol described by Hatanaka & Galetti (2004), and the 5S rDNA probe was obtained from *Leporinus elongatus* (Valenciennes, 1850) (Martins & Galetti , 1999). 18S rDNA and 5S rDNA probes were labeled with Biotin 14-dATP by nick translation following the manufacturer's instructions (Bionick Labeling System – Invitrogen). Additionally, 18S rDNA and 5S rDNA probes were also labeled with Digoxigenin 11-dUTP (Roche Applied Science) via PCR (Polymerase Chain Reaction). The hybridization signals were detected using anti-digoxigenin-rhodamine and conjugated avidin-fluorescein (FITC). Hybridization signals were amplified with biotinylated anti-avidin antibodies. The metaphasic chromosomes were treated using methods described by Pinkel *et al.* (1986), counterstained with DAPI and analyzed under optical photomicroscope (Olympus BX61). The metaphase figures were captured using the software Image Pro Plus 6.0 (MediaCybernetics).

Chromosome morphology was determined according to the arm ratio proposed by Levan *et al.* (1964). The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) and were organized in decreasing size in the karyotypes.

Molecular analysis

Total genomic DNA was isolated from the fin or muscle tissue of each specimen using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. The partial mitochondrial Cytochrome C Oxidase subunit I gene (COI - ~648 pb) was amplified by polymerase chain reaction (PCR) using two sets of primers: FishF1 - 5´TCAACCAACCACAAAGACATTGGCAC3´; FishF2 - 5´TCGACTAATCATAAAGATATCGGCAC3´; FishR1 - 5´TAGACTTCTGGGTGGCCAAAGAATCA3´ and FishR2 -

Table 1. Cytogenetic data available for species of the genus *Piabina*. References: 1 - Portella *et al.* (1988); 2 - Peres *et al.* (2007); 3 - present study; (SP) São Paulo State; (MG) Minas Gerais State.

Species	Locality	Karyotype	2n	FN	NORs	Ref.
Piabina argentea	Mogi Guaçu (SP)	26m/sm+26st/a	52	-	06	1
Piabina argentea	São Francisco (MG)	08m+14sm+16st+14a	52	90	02	2
Piabina argentea	Itatinga (SP)	04m+22sm+10s+16a	52	88	02	3
Piabina argentea	Botucatu (SP)	08m+18sm+18st+10a	52	98	02	3
Piabina argentea	Bauru (SP)	04m+24sm+10st+14a	52	90	04	3
Piabina anhembi	Salesópolis (SP)	08m+10sm+16st+18a	52	86	02	3

Fig. 1. Map indicating the collection points for *Piabina argentea* and *P. anhembi*, the drainage regions of the Parapanema and Tietê river basins are highlighted. Symbols correspond to fish samples from Bauru (square), Botucatu (circle), Itatinga (star), and the triangle represents the sample of *P. anhembi* from Salesópolis.

5´ACTTCAGGGTGACCGAAGAATCAGAA3´ (Ward *et al*., 2005). The whole Cytochrome B (CytB - ~1118 pb) mitochondrial gene was amplified by PCR using the primers CytB-F - 5´GACTTGAAAAACCAYCGTTGT3´ and CytB-R - 5´GCTTTGGGAGTTAGDGGTGGGAGTTAGAATC3'. PCR was carried out on a thermocycler (Veriti® 96-Well Thermal Cycler, Applied Biosystems) in a total volume of 12.5 µl containing 0.3 µl of dNTP (2 mM), 1.25 µl 10X Taq buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% TritonX-100 and 1.5 mM $MgCl_2$), 0.3 µl of each primer (10 µM), 0.7 µl of $MgCl_2$ (50 mM), 0.05 µl of *Taq-Pht* DNA polymerase (5 U), 1 µl of template DNA (10-20 ng) and ultrapure water. The conditions of thermocycling for the COI gene included the following: an initial denaturation at 95ºC for 5 min; 30 cycles of denaturation at 95°C for 45 seconds, annealing at 54°C for 30 seconds and extension at 72° C for 60 seconds; and a final extension at 72°C for 10 minutes. The conditions of thermocycling for gene CytB included the following: an initial denaturation at 95°C for 5 minutes; two cycles of denaturation at 95 C for 30 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 60 seconds; 2 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 45 seconds and extension at 72°C for 60 seconds; 2 cycles of denaturation at 95°C for 30 seconds, annealing at 48°C for 45 seconds and extension at 72°C for 60 seconds; 25 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 45 seconds and extension at 72°C for 60 seconds; and a final extension at 72°C for five minutes.

The amplification products were verified on a 1% agarose gel stained with Loading Dye Blue Green I (LGC Biotecnologia). PCR products were purified with the enzyme-ExoSap® IT (USB Corporation) according to the manufacturer's protocol. The purified PCR product was used as a template for the PCR sequencing of both strands of DNA. The sequencing reaction was performed using the BigDye Terminator v. 3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems) in a total volume of 7 µl DNA containing 1.4 µl template, 0.35 µl primer (10 mM), 1.05 µl of 5X buffer, 0.7 µl of BigDye mix and water. The thermal cycling conditions were an initial denaturation at 96°C

for two minutes followed by 30 cycles of denaturation at 96°C for 45 seconds, annealing at 50°C for 60 seconds, extension at 60°C for four minutes. PCR products for sequencing were purified with EDTA / sodium acetate / ethanol following the protocol suggested in the BigDye Terminator Cycle Sequencing kit v.3.1 (Applied Biosystems). All of the samples were sequenced in an automatic sequencer ABI3130 Genetic Analyzer (Applied Biosystems) following the manufacturer's instructions. All of the sequences were analyzed in the program SeqScape® v2.6 (Applied Biosystems) to obtain the consensus sequences and to check for the presence of deletions, insertions and stop-codons. Sequences were aligned using the online version of the program MUSCLE (Edgar, 2004). The genetic distances between and within the clusters observed were calculated using the K2P distance model (Kimura, 1980). The neighbor-joining tree of the K2P distance was created to provide a graphic representation of the relationships among the specimens and clusters of *P. argentea* and *P. anhembi* species using the program MEGA 4.0 (Tamura *et al*., 2007). Bootstrap resampling (Felsenstein, 1985) was applied to assess the support for individual nodes using 1000 pseudo replicates. The sequences obtained were deposited in GenBank (accession numbers: COI - HM144047, HM144048, HM144049, HM144050, HM144091, HM144085, HM144052, HM144053, HM144086, HM144084, HM144089, HM144088, HM144094, HM144095, HM144097; CytB - GU908165, GU908166, GU908167, GU108168, GU908207, GU908201, GU908170, GU908171, GU908202, GU908200, GU908205, GU908204, GU908209, GU908210, GU908212).

Results

Cytogenetic analysis

We analyzed forty-five specimens of *P. argentea* of local samples from two tributaries of the Tietê River (Botucatu and Bauru regions) and one sample from a tributary of the Paranapanema river (Itatinga region); another sample analyzed consisted of thirteen specimens of *P. anhembi* from the upper Tietê River (Salesópolis region) (Table 1; Fig. 1).

The analysis of 14 specimens of *P. argentea* (5 males and 9 females) from Itatinga (Table 1) with conventional Giemsa staining revealed a diploid number of 2n=52 (4m+22sm+10st+16a) (Fig. 2a) and a fundamental number of 88 (Table 1). Heteromorphic chromosomes were not identified in male or female karyotypes. Two subtelocentric chromosomes contained Ag-NOR marks on the terminal region of the short arms. These chromosomes are pair 18 (Fig. 2a). Fluorescent *in situ* hybridization (FISH) using the 18S DNA probe was consistent with the Ag-NOR marks (Fig. 3a), and the 5S rDNA probe marked four sites located in the terminal region on the carrier chromosomes (Fig. 3b). Heterochromatic blocks were found in the centromeric region of multiple chromosomes and in the short arms of the second submetacentric chromosome pair (Fig. 2b).

In the sample from Botucatu, 11 specimens of *P. argentea* (6 females and 5 males) were analyzed (Table 1). The analysis with

conventional Giemsa staining showed a diploid number of 2n=52 (6m+18sm+18st+10a) (Fig. 4a) and a fundamental number equal to 98 (Table 1). Heteromorphic chromosomes were not identified in male or female karyotypes. Silver marks on the terminal region of the short arm in two chromosomes, pair 14, were identified by the Ag-NORs technique (Fig. 4a), and fluorescent *in situ* hybridization using the 18S rDNA probe confirmed the Ag-NORs results and two other marks were also identified (Fig. 3c). The 5S rDNA probe was located on six chromosome sites in terminal regions (Fig. 3d). Constitutive heterochromatin is restricted to the centromeric regions of most of the chromosomes (Fig. 4b).

From the Bauru location, 20 specimens of *P. argentea* (8 females and 12 males) were analyzed. The analysis with conventional Giemsa staining showed a diploid number of 2n = 52 (4m+24sm+10st+14a) and a fundamental number of 90 in specimens from this location (Table 1). Heteromorphic chromosomes were not identified in the karyotypes of males or
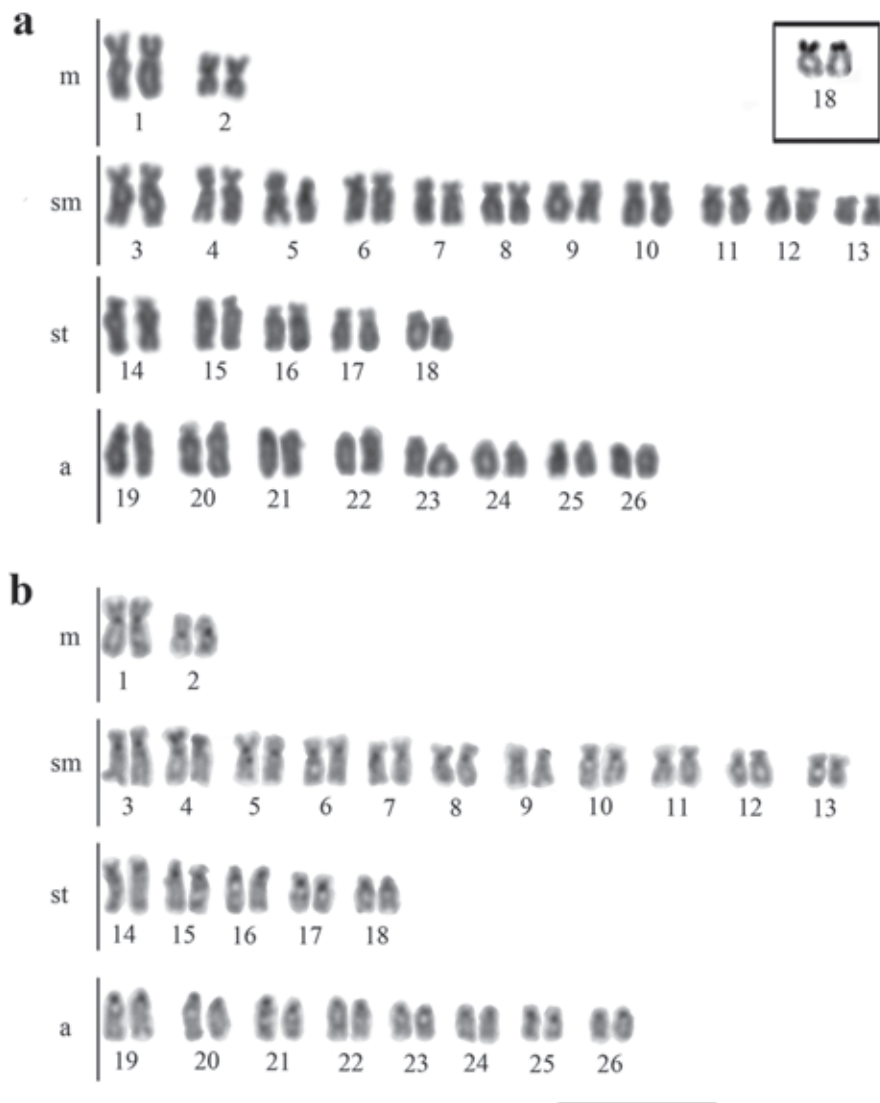

Fig. 2. Karyotype of *P. argentea* from Itatinga, after Giemsa staining (**a**) and C-banding (**b**) is shown. In detail, the NOR-bearing chromosomes are shown. Bar = 10 μm.

females. Silver marks on the terminal region of the short arms in four submetacentric chromosomes, pairs number 3 and 17, were identified using the Ag-NORs technique (Fig. 5a), and fluorescent *in situ* hybridization using the 18S rDNA probe confirmed the Ag-NORs results and two other marks were also
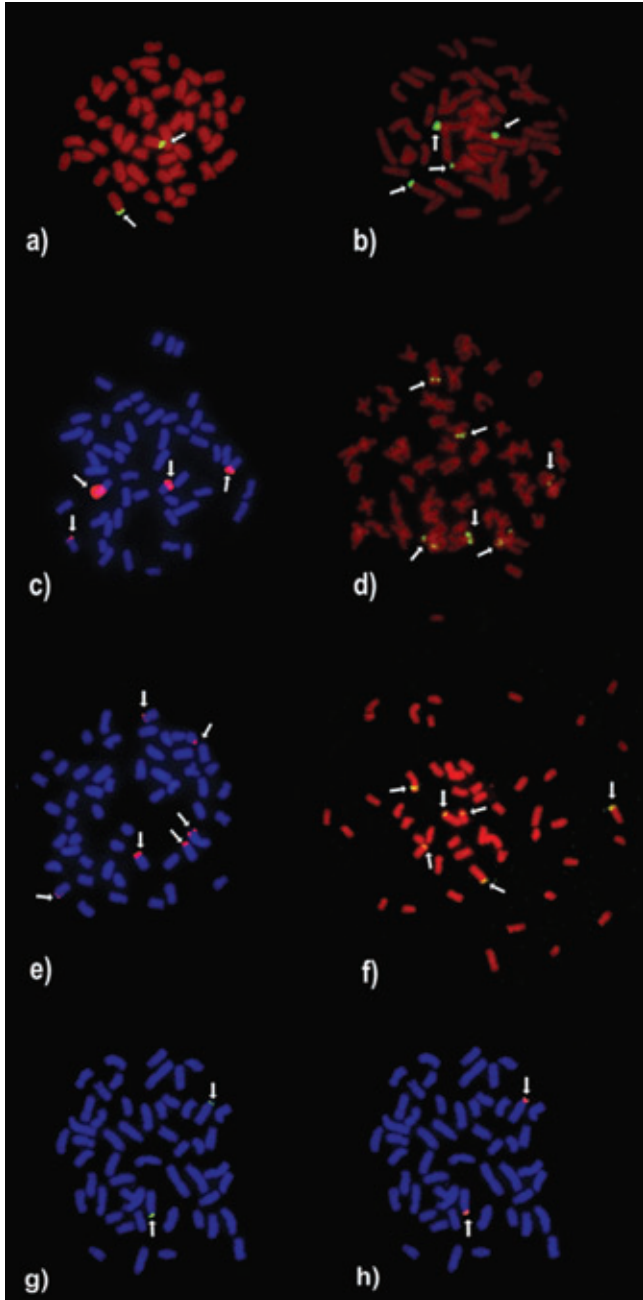

Fig. 3. Somatic metaphase of *P. argentea* are shown for the following: sample from Itatinga after *in situ* hybridization with the probes 18S (**a**) and 5S (**b**); sample from Botucatu after *in situ* hybridization with the probes 18S (**c**) and 5S (**d**); and sample from Bauru after *in situ* hybridization with the probes 18S (**e**) and 5S (**f**). Somatic metaphase of *P. anhembi* after *in situ* hybridization with the 18S rDNA probe (**g**) and 5S (**h**) are shown.

identified (Fig. 5a) (Fig. 3e). The 5S rDNA probe revealed six sites carrying the gene located in the terminal regions of the chromosomes (Fig. 3f). Conspicuous heterochromatic blocks were found in the centromeric regions in almost all of the chromosomes. Heterochromatin was located in the whole small arm of chromosomes 15 and 16 in the terminal region of chromosomes number 3, 6, 17, 20 and 22 (Fig. 5b).

The 13 specimens of *P. anhembi* (4 females and 9 males) analyzed with conventional Giemsa staining showed a diploid number of 2n=52 chromosomes (8m+10sm+16st+18a) and a fundamental number of 86 (Table 1 and Fig. 6a). Heteromorphic chromosomes were not identified in the karyotypes of males or females. Silver marks on the terminal region of the short arms of one chromosome pair, pair 21, were identified by Ag-NORs technique. A size polymorphism in the NORs was observed, mainly in pair 21 (Fig. 6a). Fluorescent *in situ* hybridization using the 18S DNA probe marked only two chromosomes, pair 21 (Fig. 3g). These chromosomes were also marked by the 5S rDNA probe (Fig. 3h), which demonstrates the synteny of the 18S and 5S rDNA regions. C-banding revealed that sharp heterochromatic blocks are located in the centromeric region of multiple chromosomes and are also located interstitially in the pericentromeric and terminal regions of some chromosomes within the karotype (Fig. 6b).

Molecular analysis

A partial sequence of the COI gene (~648 bp) and the whole sequence of the CytB gene (~1118 bp) were obtained from 11 specimens of *P. argentea* from the three samples analyzed and from four specimens of *P. anhembi*. The total sequence length obtained was 1766 nucleotides, and no insertions, deletions or stop-codons were identified.

The neighbor-joining tree of the K2P genetic distance show that cluster formations coincide with the four samples analyzed. Three clusters represent the *P. argentea* samples and one cluster represents the *P. anhembi* sample (Fig. 7). The genetic distance between clusters of *P. argentea* ranged from 2.8% (Botucatu x Itatinga) to 4.6% (Botucatu x Bauru) (Table 2). The variation within each cluster ranged from 0 (Botucatu) to 0.2% (Itatinga) (Table 2). The genetic distance (K2P) found between the *P. anhembi* cluster and the clusters of *P. argentea* ranged from 2.4% (*P. anhembi* x Itatinga) to 4.5% (*P. anhembi* x Bauru).

Discussion

A diploid number of 52 chromosomes was observed in all specimens of *P. argentea* and *P. anhembi* analyzed in this study. The same chromosome number was observed by Portela *et al*. (1988) and Peres *et al*. (2007, 2008) in their analysis of *P. argentea*, which suggests that there is a conserved diploid number for this genus. The same chromosome number is also observed in other fish species belonging to the subfamily Stevardiinae (Oliveira *et al*., 2005), which includes the genus *Bryconamericus* (Eigenmann, 1907) and *Mimagoniates* (Regan, 1907).

The various karyotypic formula and fundamental numbers found in the samples of *P. argentea* and *P. anhembi* analyzed indicate that extensive rearrangements are occurring in the chromosomes, *e.g.*, paracentric inversions that alter the karyotypic formula without causing changes in the diploid number. Stable differences in the karyotypes indicate that the different samples of *P. argentea* analyzed are reproductively isolated, which favors the establishment of the observed changes.

The nucleolus organizer regions detected by the Ag-NORs technique have been extensively studied in fish and are considered good cytogenetic markers, which support taxonomic studies (Galetti , 1998). In the samples of *P. argentea* and *P. anhembi* studied, simple (Itatinga, Botucatu and *P. anhembi*) and multiple (Bauru) NORs were detected (Fig. 5a) in chromosomes with different morphologies. These findings may be due to active chromosome modifications (Gromicho & Collares-Pereira, 2004), chromosomal translocation or to their association with
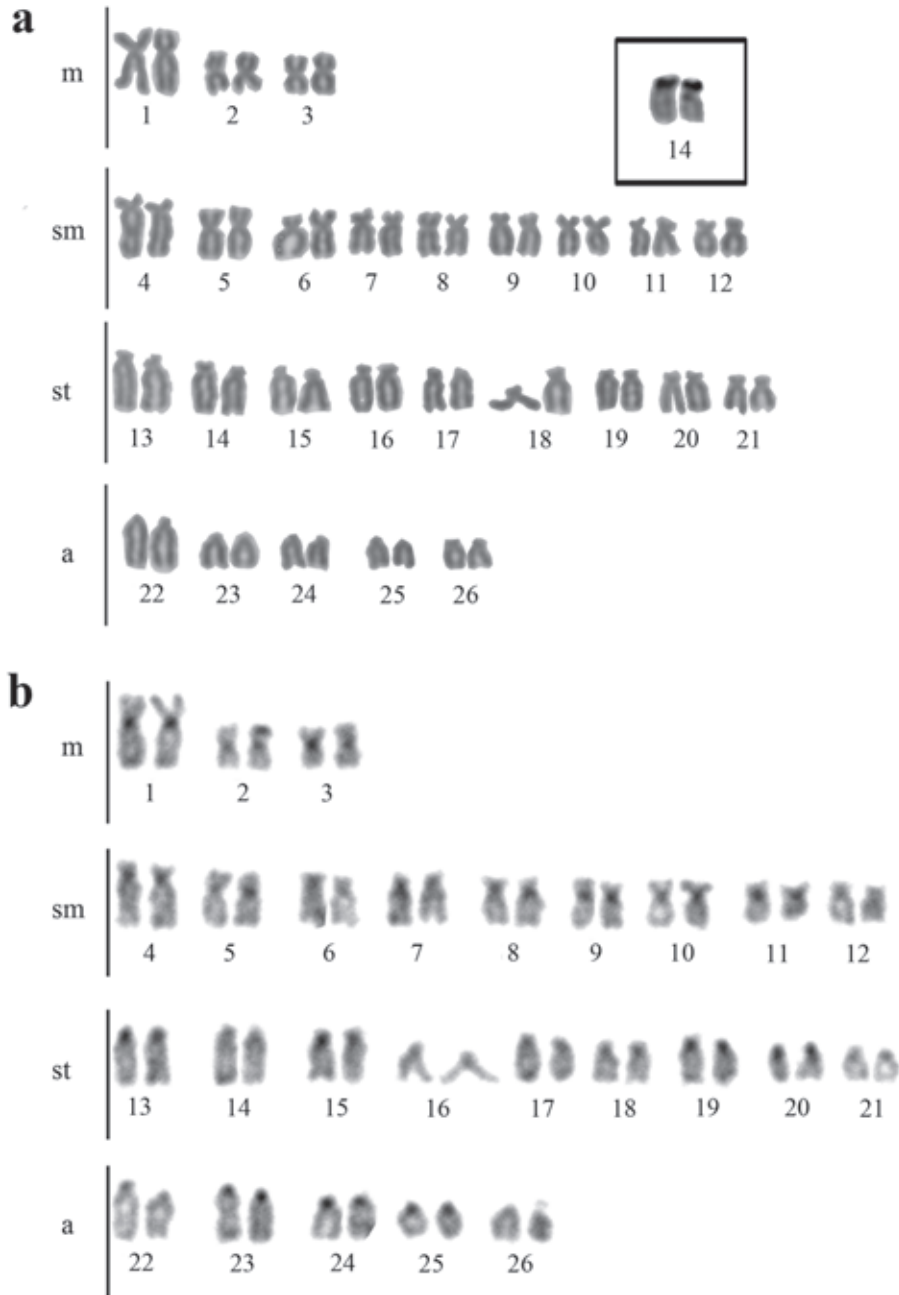

Fig. 4. Karyotype of *P. argentea* from Botucatu, after Giemsa staining (**a**) and C-banding (**b**) are shown. In detail, the NOR-bearing chromosomes are shown. Bar = 10 μm.

transposable elements that carry rDNA sequences and disperse them along the genome. The polymorphism of the NORs is a relatively common event in Neotropical fish (*e.g.* Foresti *et al.*, 1981; Brum *et al.*; 1998; Vicari *et al.*, 2006).

Multiple sites of 18S rDNA were found in the sample of *P. argentea* collected in the São Francisco River Basin and analyzed by Peres *et al.* (2008). Similar sites were also found in the population of *P. argentea* from the Bauru sample analyzed in the present work (Fig. 3e). In the samples of *P. anhembi* and *P. argentea* from Itatinga two 18S rRNA gene sites were detected and sample from Botucatu four 18S rRNA gene sites were observed (Fig. 3). *In situ* hybridization (FISH) using the 18S rDNA probe confirmed the data obtained by silver nitrate impregnation into the sample of *P. argentea* from Itatinga and the population of *P. anhembi* (Fig. 3). However, FISH revealed six chromosome sites in individuals from Bauru, two marks more

than the number visualized by Ag-NORs (Figs. 3 and 5, respectively). Four markers were visualized by FISH in the sample from Botucatu, which is two more than the observed number by silver nitrate incorporation (Figs. 3 and 4). The differences in marking by these techniques may reflect the activity of the NORs during the previous interphase (Roussel *et al.*, 1996; Zurita *et al.*, 1997; Gromicho & Collares-Pereira, 2004).

Silver has an affinity for some proteins associated with NORs and not with rDNA, which may produce false results depending on the activity of the ribosomal gene. The use of *in situ* hybridization should reveal a more accurate number of rDNA sites present in the genome. Recently, it has been suggested that filamentous fungi are capable of multiplying and integrating rDNA sites into other areas of the genome through a process of retrotransposition (Rooney & Ward, 2005). The intra and interspecific variation in the rDNA site locations
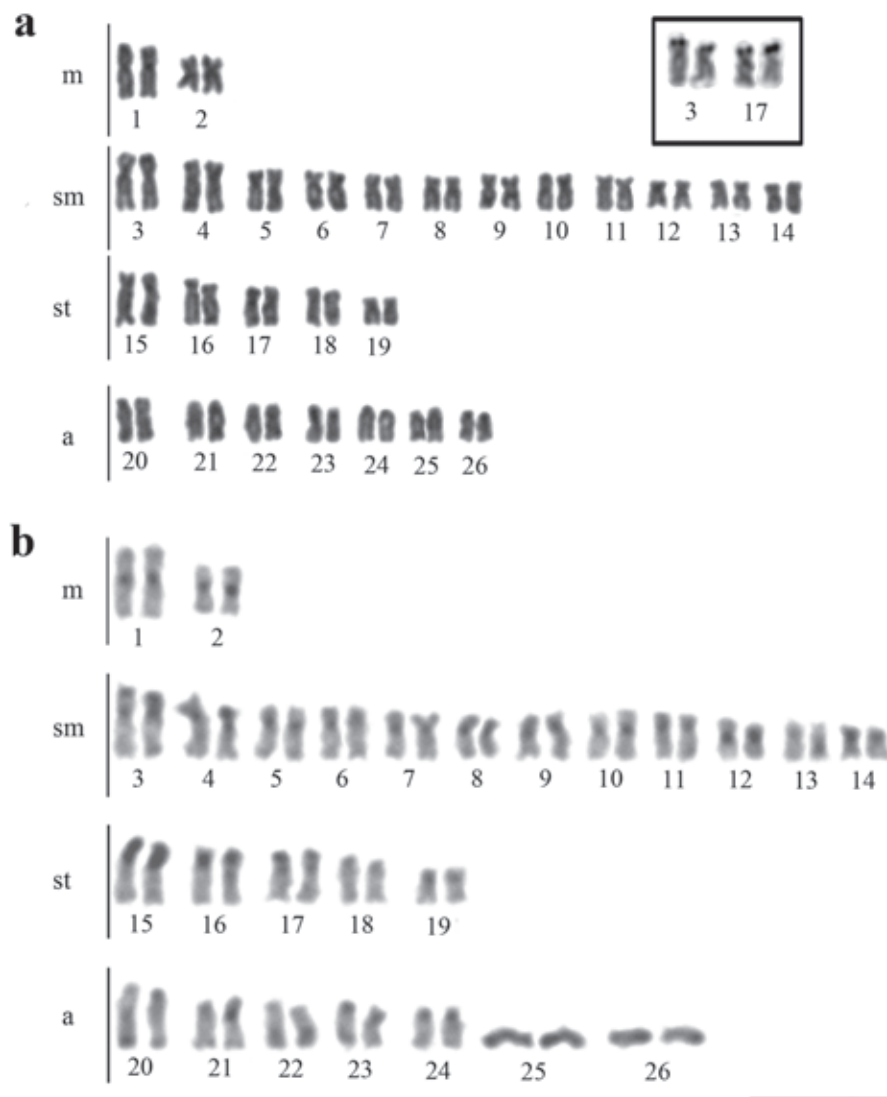

Fig. 5. Karyotype of *P. argentea* from Bauru, after Giemsa staining (**a**) and C-banding (**b**) are shown. In detail, the NOR-bearing chromosomes are shown. Bar = 10 μm.

observed in grasshoppers may be the result of the transposition of a few rRNA genes to new chromosome locations. After their amplification, and in case of the elimination of the original NOR, the new site could substitute for the original site in function. The inactive rDNA loci observed may correspond to those sites that are in the process of being eliminated, and the cryptic NORs may correspond to a few rDNA units that have been moved but not yet amplified (Cabrero & Camacho, 2008).

The next step should be to determine if rDNA is present in these cryptic NORs and to investigate whether they are associated with transposable elements that move along with them through genomes. In species showing interpopulation variation in rDNA location, such as *Eyprepocnemis plorans* (Charpentier, 1825), a spread of rDNA throughout the genome seems to have recently taken place (Cabrero *et al*., 2003). Guillén *et al*. (2004) worked with humans and chimpanzees and suggested that the elimination of rDNA or gene silencing by methylation is caused by the presence of heterochromatin, which is involved in different mechanisms that inactivate

NORs. Cabrero & Camacho (2008) proposed that the NORs may correspond to hidden springs, *i.e.*, a gene with a few copies of rRNA that moved to a new location, and the inactive rDNA loci could be in the process of being eliminated. Many hidden loci could occur with the incorporation of rRNA genes into new chromosome locations, and its expansion would give rise to new NORs (Singh *et al*., 2009).

The 5S rDNA clusters have been identified in two pairs of chromosomes of *P. argentea* by Peres *et al*. (2008), and this finding is consistent with the data obtained for the *P. argentea* sample from Itatinga (Fig. 3b). However, in *P. argentea* individuals from the Botucatu and Bauru samples six chromosomes were found to carry the genes (Fig. 3d and 3f), and in *P. anhembi* only one chromosome pair contained 5S rDNA clusters. This variation in the number of chromosomes carrying 5S rDNA may be due to an association between these regions and transposons, which are responsible for scattering rDNA regions in the genome of eukaryotes (Drouin & Moniz de Sá, 1995). The variations found in this region have been a
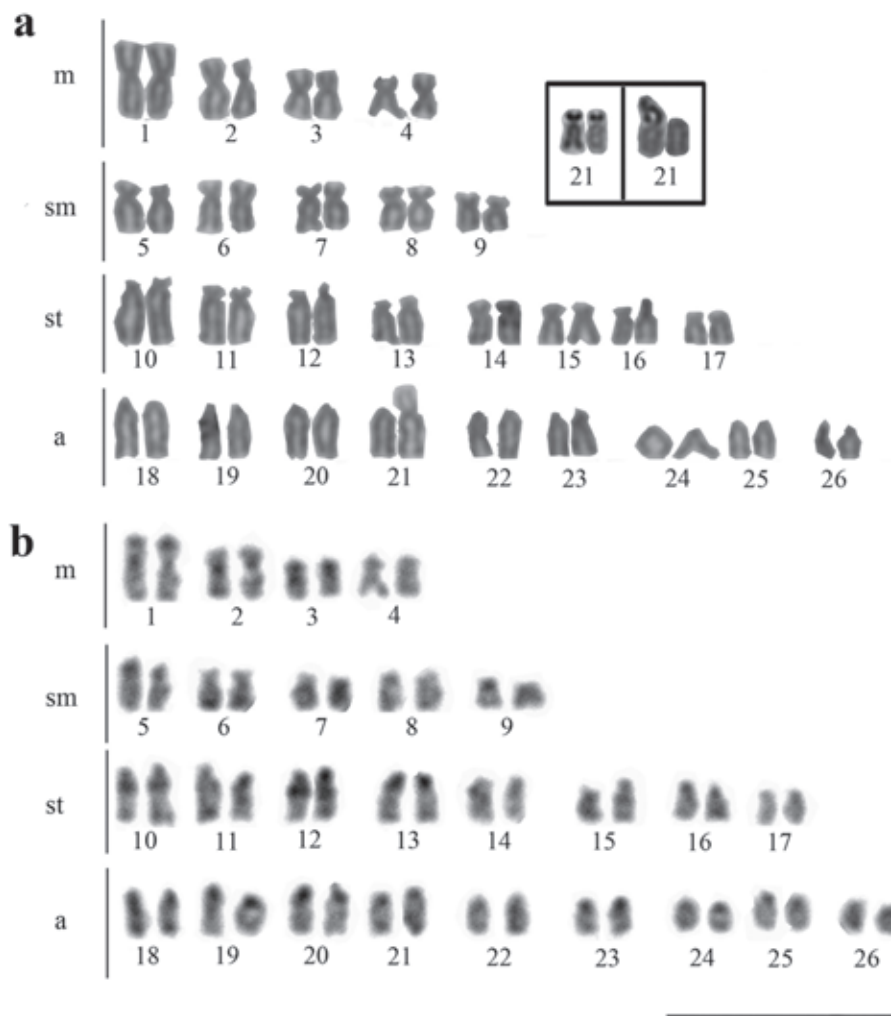

Fig. 6. Karyotype of *P. anhembi* from Salesópolis, after Giemsa staining (**a**) and C-banding (**b**) are shown. In detail, the NOR-bearing chromosomes are shown. Bar = 10 µm.
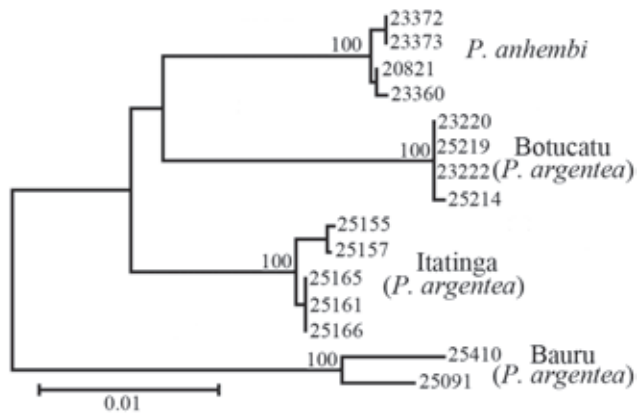
Fig. 7. Neighbor-joining cladogram constructed from the partial sequences of genes COI and CytB in the species *P. argentea* and *P. anhembi* are shown. Bootstrap with 1000 replicates.

Table 2. Table of genetic distance (K2P) to COI and CytB genes in one population of *Piabina anhembi* and three populations of *P. argentea* (Bauru, Itatinga and Botucatu) analyzed.

	Bauru	Itatinga	*Piabina anhembi*	Botucatu
Bauru	0.001			
Itatinga	0.040	0.002		
Piabina anhembi	0.045	0.024	0.001	
Botucatu	0.046	0.028	0.027	0.000

very useful tool for identifying different populations, as demonstrated in studies on the genus *Astyanax* (Baird & Girard, 1854) (Ferro *et al.*, 2001; Almeida-Toledo *et al.*, 2002; Mantovani *et al.*, 2005; Vicari *et al.*, 2008). The location of the 5S rDNA region in chromosomes is described for more than 60 species belonging to seven fish orders. These studies have revealed interesting information about the organization and structure of the 5S rDNA sequences present in fish chromosomes (Martins & Wasko, 2004). In many vertebrates the 5S rDNA gene is located in only one chromosome pair (Suzuki *et al.*, 1996; Mäkinem *et al.*, 1997). Even the mapping of the 5S rDNA sites in fish has demonstrated that sites are frequently located at a single chromosome locus, which may correspond to an ancestral condition in this group (Martins & Galetti, 1999). In amphibians (Schmid *et al.*, 1987; Lucchini *et al.*, 1993) and fishes (Mazzei *et al.*, 2004; Nirchio & Oliveira, 2006) the 5S rDNA genes can be located in one or more chromosomes. Variations in the 5S rDNA gene location may occur due to the presence of pseudogenes, insertions, deletions and repetitions, which have often been characterized in various organisms (Suzuki *et al.*, 1996; Sadjak *et al.*, 1998; Alves-Costa *et al.*, 2006).

The occurrence of 18S and 5S ribosomal DNA sites in synteny is rare in vertebrates and has only been reported in a few organisms, such as the fish species *Salmo salar* (Linnaeus, 1758) (Pendás *et al.*, 1994), *Oncorhynchus mykiss* (Walbaum, 1792) (Moran *et al.*, 1996), *Prochilodus argenteus* (Hatanaka & Galetti, 2004), *Prochilodus lineatus* (Valenciennes, 1836) (Jesus *et al.*, 2003; Vicari *et al.*, 2006), five species of the genus *Astyanax* (Almeida-Toledo *et al.*, 2002) and amphibians (Lucchini *et al.*, 1993). Moreover, the *loci* have been mapped on different chromosomes in several fish species (Morán *et al.*, 1996; Wasko *et al.*, 2001), a condition often found in vertebrates (Lucchini *et al.*, 1993; Suzuki *et al.*, 1996). Our data show that in *P. anhembi* the 5S ribosomal site is syntenic with the 18S rDNA site, which is located subterminally on the short arm of the chromosome pair 21 (Fig. 3). The synteny occurrence

of these genes may be due to a risk of sequence loss in these regions by chromosomal deletions during the moving process to other chromosome sites (Hatanaka & Galetti, 2004).

Previous studies using the C-banding technique (BSG technique) have shown that some species of fish (Gold *et al.*, 1990), as well as other animals and plants (Sumner, 1990), have heterochromatic bands in different quantities, which are mainly distributed in the centromeric or telomeric regions of chromosomes and less frequently in interstitial regions (Weiler & Wakimoto, 1995; Oliveira & Wright, 1998). All of the *P. argentea* samples analyzed contained heterochromatic blocks in the centromeric region of almost all of the chromosomes and in the terminal regions of some of the chromosomes. *P. argentea* specimens from Itatinga are partially heterochromatic in the short arm of chromosome pair 4 (Fig. 2b). In individuals from the Botucatu sample the short arms of chromosome pairs 13 and 15 are partially heterochromatic. In individuals from the Bauru sample the short arm of chromosome pairs 3, 4 and 17 are partially heterochromatic, while in chromosome pairs 15 and 16 the short arms were entirely heterochromatic. Additionally, in this sample chromosomes pairs 20 and 22 show a marked block in the terminal region of the long arm. The *P. anhembi* specimens contained bands in the interstitial region of the long arms in chromosome pairs 10 and 12.

The differences in the distribution of heterochromatin in the karyotypes of the *P. argentea* analyzed provide strong evidence that these samples may represent different groups. The results showing that *P. anhembi* contains bands in different regions than the bands observed in *P. argentea* demonstrates that the pattern of heterochromatin distribution can be a useful tool in the cytogenetic studies of several Neotropical fish groups. The differences in the distribution of C-banding positive segments may be used to help characterize of genera, species and populations (Mantovani *et al.*, 2000). Although the functional role of heterochromatin is still poorly understood, it appears that the expression of some "euchromatic" genes is dependent on both "heterochromatic" gene expression and the activation of inhibitory factors (Weiler & Wakimoto, 1995; Oliveira & Wright, 1998).

According to the molecular analyses, *P. argentea* could be divided into three groups that correspond to the samples analyzed. The genetic distance (K2P) between the clusters of *P. argentea* ranged from 2.8% (Botucatu x Itatinga) to 4.6%

(Botucatu x Bauru) (Table 2). The variation within each cluster ranged from 0 (Botucatu) to 0.2% (Itatinga) (Table 2), and the genetic distance (K2P) between the clusters of *P. anhembi* and *P. argentea* ranged from 2.4% (*P. anhembi* x Itatinga) to 4.5% (*P. anhembi* x Bauru). These results suggest that the three groups analyzed represent distinct taxonomic units, and this finding is supported by the similar values in genetic divergence (K2P) found between the groups of *P. argentea* and *P. anhembi* (Table 2), which was used as a standard reference to separate the species.

In a recent review on the distribution of genetic divergence in the "Barcoding" data of fishes (N = 1088), Ward (2009) showed that approximately 17% of the genetic divergence values among congener species is less than 3% and 3.7% of the congener comparisons are less than 1%. The author suggests that if an unknown sample has a genetic divergence greater than 2% to a known specimen, the probability that the sample is a different species exceeds 95%. Studies in species of the genus *Astyanax* from Mesoamerica performed by Ornelas-Garcia *et al.* (2008) revealed similar results. The authors suggested that the groups studied represent new species of *Astyanax* (Ornelas-Garcia *et al.*, 2008). Similar results in genetic divergence inter-clusters to those found in the present work suggest that more than one species exists under the same denomination (Smith *et al.*, 2005; Witt *et al.*, 2006; Ward *et al.*, 2007; Ward *et al.*, 2008; Nguyen & Seifert, 2008; Yassin *et al.*, 2008).

The cytogenetic and molecular differences found in the samples of *Piabina* analyzed support the hypothesis proposed by Lowe Mc Connell (1999) that large tropical rivers may act as "barriers" (physical, chemical or biotic) for small fish species and contribute to their speciation because of their isolation in micro-basins. These events appear to have occurred with the *P. anhembi* that are only found in the high Tietê River basin. The separation of small groups with little to no gene flow would help to maintain the different characteristics found in the separated groups, and this type of separation could explain the cytogenetic and mitochondrial DNA differences observed in this study. Our data emphasizes the importance of combining both cytogenetic data and molecular markers in evolutionary studies. Our data also assists in the genetic identification of *Piabina* species. Furthermore, the use of mitochondrial genes in systematic and evolutionary studies has become important in helping to distinguish species. We conclude that the samples of *P. argentea* analyzed may represent three different biological units that have not yet been nominated and suggest isolation mechanisms that have led to the initial differentiation of these groups.

Acknowledgements

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