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Cytogenetic and molecular analyses in troglobitic and epigean species of *Pimelodella* (Siluriformes: Heptapteridae) from Brazil

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Samples from seven different locations of the genus *Pimelodella* were genetically examined, two caves (exclusively subterranean, upper Tocantins River and São Francisco River) and five epigean (from upper Paraná River basin). Cytogenetic analyses revealed the same diploid number (2n=46) for all species besides similarities in both number and location of nucleolar organizer regions and C bands. FISH with 5S rDNA probes and CMA_3 staining indicated significant differences among the studied species. Application of PCR-RFLP in ATPase 6 and 8 mitochondrial genes allowed building a minimum evolution phenogram identifying the close evolutionary relationship among groups. Both chromosomal and molecular data were useful to infer the relationships among studied *Pimelodella* species.

Amostras de sete diferentes localidades do gênero *Pimelodella* foram geneticamente analisadas, duas cavernícolas (exclusivamente subterrâneas, alto rio Tocantins e rio São Francisco) e cinco epígeas (provenientes da bacia do alto Paraná). Análises citogenéticas revelaram o mesmo número diploide (2n=46) para todas as espécies, além de similaridades no número e localização das regiões organizadoras de nucléolo e bandas C. FISH com sondas de rDNA 5S e marcação com CMA_3 indicaram diferenças significativas entre as espécies estudadas. A aplicação da técnica de PCR-RFLP nos genes mitocondriais ATPase 6 e 8 permitiu a construção de um fenograma de evolução mínima identificando uma estreita relação evolutiva entre as espécies estudadas.

Key words: Cave fish, Cytogenetics, Evolutionary relationships, FISH, PCR-RFLP.

Introduction

The neotropical region encompasses the richest and most diversified fish fauna in the world (Nelson, 2006). Characiformes and Siluriformes are the best represented fish orders in number of species throughout this region. Within the family Heptapteridae (Siluriformes), *Pimelodella* is the most specious genus, with more than 70 nominal species, among which at least 30 occur in Brazil (Bockmann & Guazzelli, 2003). These are small to medium sized catfishes (most with less than 15 mm SL, a few surpassing 20 mm SL), generalized in morphology and habits, living in a variety of habitats, from headwater streams to large rivers, in the epigean (surface) and subterranean environment. It includes two exclusively subterranean species (troglobites, characterized by a reduction of eyes and pigmentation - *Pimelodella kronei* and *P. spelaea*) and some troglophilic populations (*e.g.*, *P. laurenti*, from Morena cave, Cordisburgo Co. - Trajano *et al*., 2009).

The lack of specializations within *Pimelodella* hampers the distinction among species based on traditional morphological taxonomic characters. The hypothesis on the basis of only two apomorphies in the caudal skeleton (innermost caudal rays not directly articulated with the hypural plates; membranes between inner caudal rays extending till their basal halves) was corroborated by Bockmann & Miquelarena (2008) who added the presence of six branchiostegal rays to the diagnosis of this clade. So far there

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is no clear cut feature that allows diagnosing a monophyletic *Pimelodella* exclusive of *Brachyrhamdia*. Even the affinities between *Pimelodella* and *Brachyrhamdia* deserve further investigation, since the characters supporting such a hypothesis are highly variable and have a high rate of homoplasy (Bockmann, pers. comm.). Especially in cases like this, taxonomic resolution may depend on non morphological sources, like cytogenetic and molecular characters, once informative at the level in question (i.e., varying among taxa).

Previous cytogenetic studies on *Pimelodella* catfishes indicated a remarkable interspecific and intraspecific karyotype variation, with diploid numbers ranging from 2n = 46 to 2n = 58 and a higher frequency of 2n = 46 (Garcia & Almeida-Toledo, 2010; Swarça *et al*., 2000). Garcia & Almeida-Toledo (2010) analyzed specimens of *Pimelodella* sp. from rio Pardo Basin, Cardoso/SP, and detected an inter individual variation concerning the presence of four small supernumerary chromosomes. In that study, the authors also reported a XX/XY sex chromosome system and a heterochromatin polymorphism in *P. boschmai* from Araras, SP, a species included in the present molecular analysis.

Nucleolar organizer regions (NORs) in this fish group have been detected by silver nitrate, chromomycin A_3 staining and flurorescent *in situ* hybridization *(FISH)* with 18S rDNA probes (Vidotto *et al*., 2004; Swarça *et al*., 2003; Garcia & Almeida-Toledo, 2010). These studies show that single NORs, usually located on short arms of submetacentric chromosomes, are predominant in this genus.

Analyses of heterochromatin distribution in *Pimelodella* revealed a preferential location at the pericentromeric region of most chromosomes (Swarça *et al*., 2003; Vissoto *et al*., 1999, 2003, 2004). Besides this pattern, Vasconcelos & Martins-Santos (2000) observed a size heteromorphism between C bands close to the pericentromeric region of the 12th chromosomal pair in populations from Paraná River. In addition, other study reported a polymorphism of heterochromatin in the genus as well: Garcia & Almeida-Toledo (2010), analyzing *P. boschmai* from Araras, SP, detected intrapopulation variation of heterochromatin segments at the terminal region of long arms in one homologous of the first chromosomal pair, restricted to the males in the studied population.

Studies involving *in situ* hybridization with 5S rDNA probes are scarce in Siluriformes. Recently, Garcia & Almeida-Toledo (2010) performed this analysis on five species of *Pimelodella* (*P. boschmai*, *P. meeki*, *P. lateristriga*, *P. gracialis*, and *Pimelodella* sp.) and reported a high numerical and positional variation of 5S rDNA sites, variation not observed for others heptapterids genus as *Rhamdia* (Garcia *et al*., 2010).

DNA molecular markers have been successfully applied on natural fish populations to establish genetic and evolutionary relationships within groups, besides assisting species conservation (Triantafylli, 1999; Mohindra, 2007). Amongst the molecular techniques related to mitochondrial DNA is quite useful since it provides information on levels of intra and interspecific polymorphisms, thereby allowing defining evolutionary relationships among studied groups (Moysés & Almeida-Toledo, 2002).

Species assigned to the genus *Pimelodella* have also been focused by molecular studies. Martim & Bermingham (2000) analyzed *Pimelodella chagresi* in order to identify differentiated haplotypes for sequencing by using PCR-RFLP of ATPase 6 and 8 genes from mitochondrial DNA. The authors verified that the nominal species *Pimelodella chagresi* encompassed a wide haplotypic variation. Almeida & Sodré (2003) used Random Amplification of Polymorphic DNA (RAPD) to assess the degree of genetic similarities in species of the genus *Pimelodus*.

The goal of the present work is to characterize some species of *Pimelodella* based on cytogenetic assays and the molecular marker PCR-RFLP. A previous cytogenetically studied species from Araras, SP (*Pimelodella boschmai*, Garcia & Almeida-Toledo, 2010) was also included to verify whether heterochromatin polymorphism is related to distinct restriction patterns via PCR-RFLP or not. The present study also aims to identify possible intra and interspecific variation and provide useful information for taxonomic and conservation studies in these species.

Material and Methods

Samples from seven populations of the genus *Pimelodella* were analyzed: Araras, SP (LIRP 8141), Botucatu, SP (LIRP 8146), São Domingos, GO (LIRP 8160), Cordisburgo, MG (LIRP 8153), Colina, SP (LIRP 8143), Guapiara, SP (LIRP 8145), and Pirassununga, SP (LIRP 8147) (Table 1). These species have been identified at Ichthyology Laboratory of Universidade de São Paulo - Ribeirão Preto (LIRP/FFCLRP) by Prof. Dr. Flávio Bockmann. After identification, these species were deposited in the collection of this laboratory.

Specimens from Araras, SP were cytogenetically studied (Garcia & Almeida-Toledo, 2010) and this population was used in the present study for molecular analyses in order to compare their taxonomic status and cytogenetic data to others *Pimelodella* sp. populations.

Cytogenetic analyses

Mitotic chromosomes were obtained from kidney cells, following the technique by Gold *et al.* (1990) adapted for fish studies. Chromosomal morphology was determined according to the arm ratio as proposed by Levan *et al.* (1964). The fundamental number (FN) was calculated taking into account that metacentric (m), submetacentric (sm) and subtelocentric (st) chromosomes are bi-armed while acrocentric (a) chromosomes are one-armed. The nucleolar organizer regions were identified by silver nitrate staining, as described by Howell & Black (1980), and chromomycin A_3 staining was performed according to Schmid (1980). Constitutive heterochromatin segments were visualized by

Table 1. Individuals analyzed in the present study, with their respective habitats, localities and hydrographic basins. Moreover, the diploid numbers and the karyotype formulae of each species are shown. "?" indicate juvenile individuals of unidentified sex (* = Garcia & Almeida-Toledo, 2010).

Species	Habitats	Number of individuals	Localities	Hydrographic Basins	2N	Karyotype Formulae
Pimelodella sp.	Surface	50 (23♂, 27♀)*	Araras - SP 22º22.994' 47º25.825'	(upper Paraná) Mogi-Guaçu	46*	38M +8SM *
Pimelodella sp.	Surface	23 (13♀, 10♂)	Botucatu - SP 22º52.081' 48º22.270'	(upper Paraná) Tietê	46	28M + 12SM + 6ST
Pimelodella spelaea	Subterranean environment	5 (3♀, 2♂)	S. Domingos - GO 22º53.491' 44º16.772'	alto Tocantins	46	26M + 16SM + 4ST
Pimelodella laurenti	Subterranean environment	10 (1♀, 9♂)	Cordisburgo - MG 19º07.296' 44º21.124'	São Francisco	46	28M + 14SM + 4ST
Pimelodella sp.	Surface	2 (2♀)	Colina - SP 20º44.635' 48º34.334'	(upper Paraná) Pardo	46	28M + 12SM + 6ST
Pimelodella sp.	Surface	5 (5♀)	Guapiara - SP 24º01.337' 48º34.262'	(upper Paraná) Paranapanema	46	26M + 16SM + 4ST
Pimelodella sp.	Surface	18 (11♀, 4♂, 3?)	Pirassununga - SP 21º55.558' 47º22.195'	(upper Paraná) Mogi-Guaçu	46	28M + 14SM + 4ST

C banding (Sumner, 1972). Fluorescent *in situ* hybridization (FISH) with 5S and 18S rDNA probes were performed according to Martins & Galetti Jr. (1999) and Hatanaka & Galetti Jr. (2004), respectively, based on the procedure by Pinkel *et al*. (1986).

Molecular Analyses

Total DNA was isolated from small pieces of fins, hepatic or muscle tissue, according to the protocol of saline extraction described by Aljanabi & Martinez (1997). The region that encompasses APTase 6 and 8 genes in the mitochondrial DNA (mtDNA) was amplified via Polymerase Chain Reaction (PCR) using primers *L8331* (5'- AAA GCR TYR GCC TTT TAA GC 3') and *H9236* (5'- GTT AGT GGT CAK GGG CTT GGR TC 3') (Perdices *et al.,* 2002). The PCR steps: 94ºC (4 min), 35 cycles at 92ºC (1 min), 58 ºC (1min), 72ºC (1min), followed by a final extension at 72ºC (10min) and cooling at 4ºC. To perform PCR-RFLP technique, the amplified products were digested with the following restriction enzymes *Alu* I, *Hae* III, *Hha* I, *Hinf* I, *Hpa* II, and *Rsa* I. The digestion products were separated by electrophoresis in 2% agarose gel stained with ethidium bromide and then photographed. Each digestion profile from each enzyme was referred as a capital letter in order of appearance, inasmuch as the composed haplotype was named with a letter code taking into account each restriction enzyme separately. Based on the digestion profiles we constructed the restriction maps for each haplotype and a matrix of presence/absence of restriction sites was generated from the restriction patterns. Using this matrix, we calculated the values of nucleotide and haplotype divergence among species, according to Nei & Tajima (1981) model using the software REAP (Restriction Enzyme Analysis Package) (McElroy *et al*., 1992). Based on these data, a minimum evolution phenogram was built using the software MEGA 4.0 (Kumar *et al*., 2007) in order to determine the relationships among species and populations.

Results

Cytogenetic Analyses

All studied species presented a diploid number of 2n = 46 and FN = 92, but the chromosomal structure varied among species (Fig. 1 and Table 1). After silver nitrate staining, a single NOR bearing pair was identified in all species, with marks on short arms of a submetacentric pair, characterizing a single NOR system (Fig. 1: box). Chromomycin A_3 staining revealed conspicuous signals coincident with NORs in all analyzed species (Fig. 1: box). In samples of Guapiara, additional weaker fluorescent marks were also observed in another submetacentric pair, equivalent to heterochromatin blocks (Fig. 2: e2). After FISH with 18S rDNA, we observed, as expected, fluorescent signals coincident with Ag-NORs besides a size heteromorphism between homologous (Fig. 1, box). In samples of Guapiara, additional fluorescent signals that could be related to the other GC rich sites were not identified.

C banding revealed small amounts of constitutive heterochromatin in the analyzed species, with marks restricted to pericentromeric position of some chromosomes. All species presented heterochromatic NORs (Fig. 2).

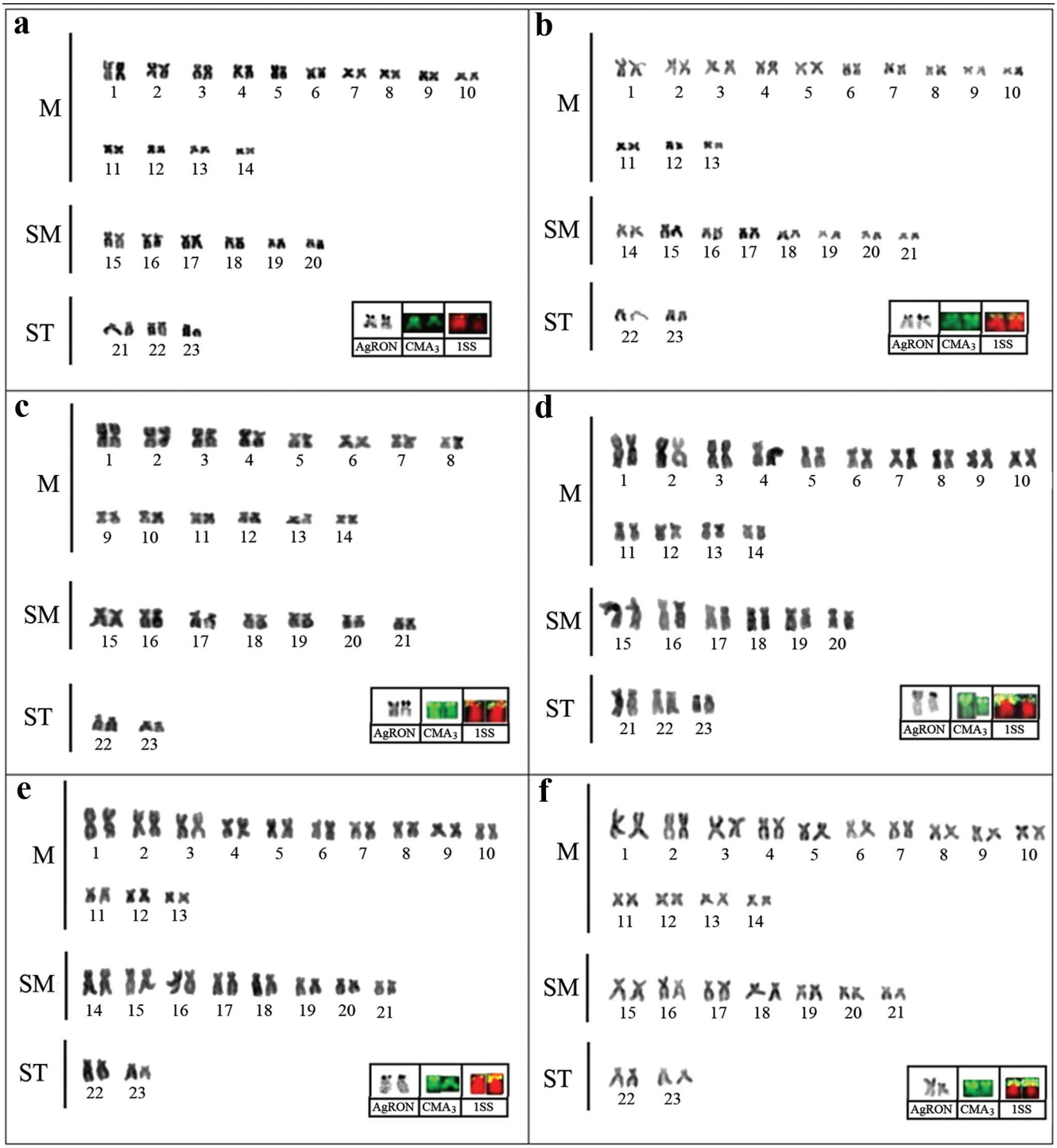

Fig. 1. Karyotypes of analyzed individuals. In detail, the silver-stained, CMA_3^+ and 18S rDNA-FISH nucleolus organizer regions are shown. **a** = *Pimelodella* sp. (Botucatu, SP); **b** = *Pimelodella spelaea* (São Domingos, GO); **c** = *Pimelodella laurenti* (Cordisburgo, MG); **d** = *Pimelodella* sp. (Colina, SP); **e** = *Pimelodella* sp. (Guapiara, SP); **f** = *Pimelodella* sp. (Pirassununga, SP).

After FISH using 5S rDNA probes in *Pimelodella laurenti* and *Pimelodella* sp. samples of Colina, SP, Guapiara, SP and Pirassununga, SP, we observed a pair of fluorescent signals located on short arms of a submetacentric chromosomal pair (Fig. 3 c, d and f). Two pairs of fluorescent signals were identified in samples of Botucatu, SP and *Pimelodella spelaea*, comprising short arms of a submetacentric pair and a large metacentric pair in samples of Botucatu, SP and short arms of two submetacentric pairs in *Pimelodella spelaea* (Fig. 3 a and b). Silver nitrate staining performed in FISH slides confirmed that 5S rDNA and NORs are not syntenic in the studied species (data not shown).

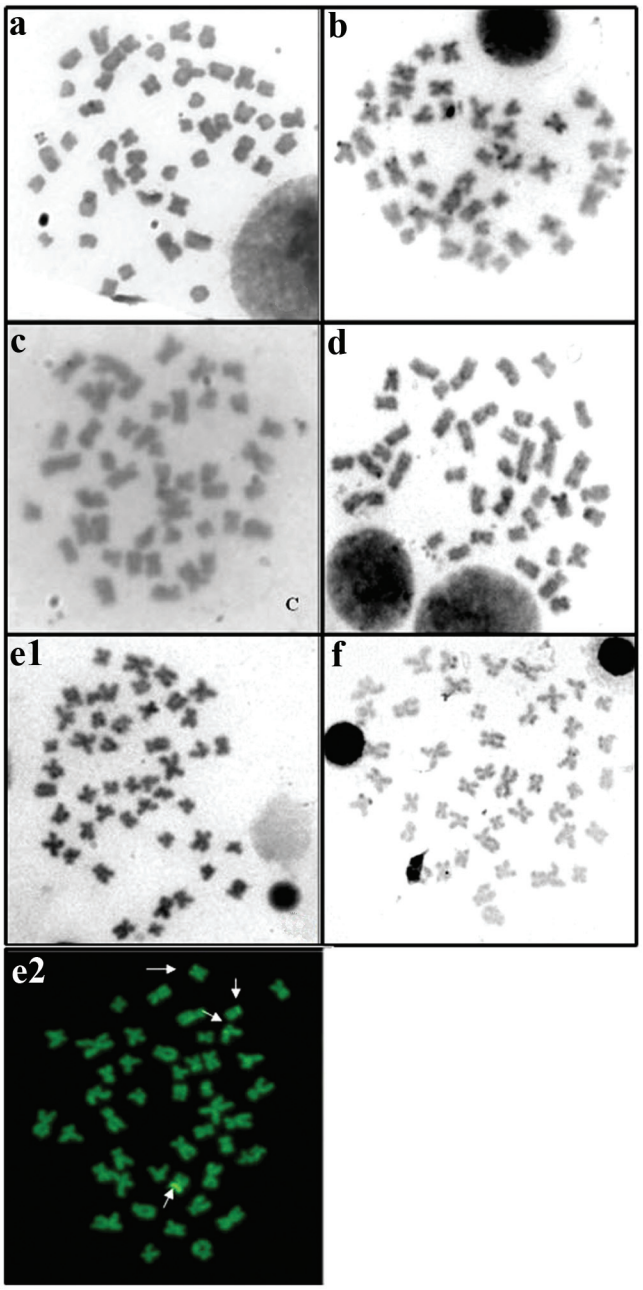

Fig. 2. Metaphases of **a** = *Pimelodella* sp. (Botucatu, SP); **b** = *Pimelodella spelaea* (São Domingos, GO); **c** = *Pimelodella laurenti* (Cordisburgo, MG); **d** = *Pimelodella* sp. (Colina, SP); **e** = *Pimelodella* sp. (Guapiara, SP); **f** = *Pimelodella* sp. (Pirassununga, SP) after CMA_3 staining, showing coincident marks with heterochromatin blocks.

Molecular Analyses

Haplotype profiles were established for the studied species based on PCR-RFLP markers (Fig. 4 and Table 2). No correspondence was observerd between haplotypic patterns and the heterochromatin polymorphism detected by Garcia & Almeida-Toledo (2010) in samples of Araras, SP.

Based on haplotype profiles of each species (Table 2), we calculated the nucleotide divergence within and among species (Table 3). A phenogram of minimum evolution was built, in which two branches can be observed. The branch number 1 was composed by the *Pimelodella* sp. populations: Araras, SP, Colina, SP and Pirassununga, SP and these populations were more closely related with Guapiara, SP and Botucatu, SP. The branch number 2 clustered the troglobitic species *Pimelodella spelaea* and *Pimelodella laurenti* (Fig. 5).

Discussion

The analyzed species presented the same diploid number of 2n = 46, a frequent condition in the genus *Pimelodella*. Yet, the chromosomal structure varied, three karyotype formulae were observed among the six species. Karyotype features of cave *Pimelodella* species, *Pimelodella spelaea* and *Pimelodella laurenti* from are herein described for the first time. The karyotype formulae of both troglobitic species differed in relation to a single chromosomal pair, as happened for *Pimelodella* sp. populations. As no changes were observed in diploid and fundamental number, these differences may be related to pericentric inversions, one of the most important chromosomal rearrangement in Heptapteridae karyotypic evolution.

Similarities between cytogenetic data of epigean and troglobitic fish forms have already been described by Almeida-Toledo *et al.* (1992) that carried out cytogenetic studies in specimens of *Pimelodella kronei* and its putative sister species *Pimelodella transitoria* collected in three caves in the State of São Paulo. Both species presented the same karyotype formulae and shared identical patterns of C bands and number and location of NORs, however, a supernumerary microchromosome in one individuals of *Pimelodella kronei* was identified.

The available reports about NORs in species of the genus *Pimelodella* indicate that most of them present single NORs located on short arms of submetacentric chromosomes, similarly to the results from the present study (Swarça *et al*., 2003; Vidotto *et al*., 2004; Garcia & Almeida-Toledo, 2010). Analyses in *Pimelodella* sp. from

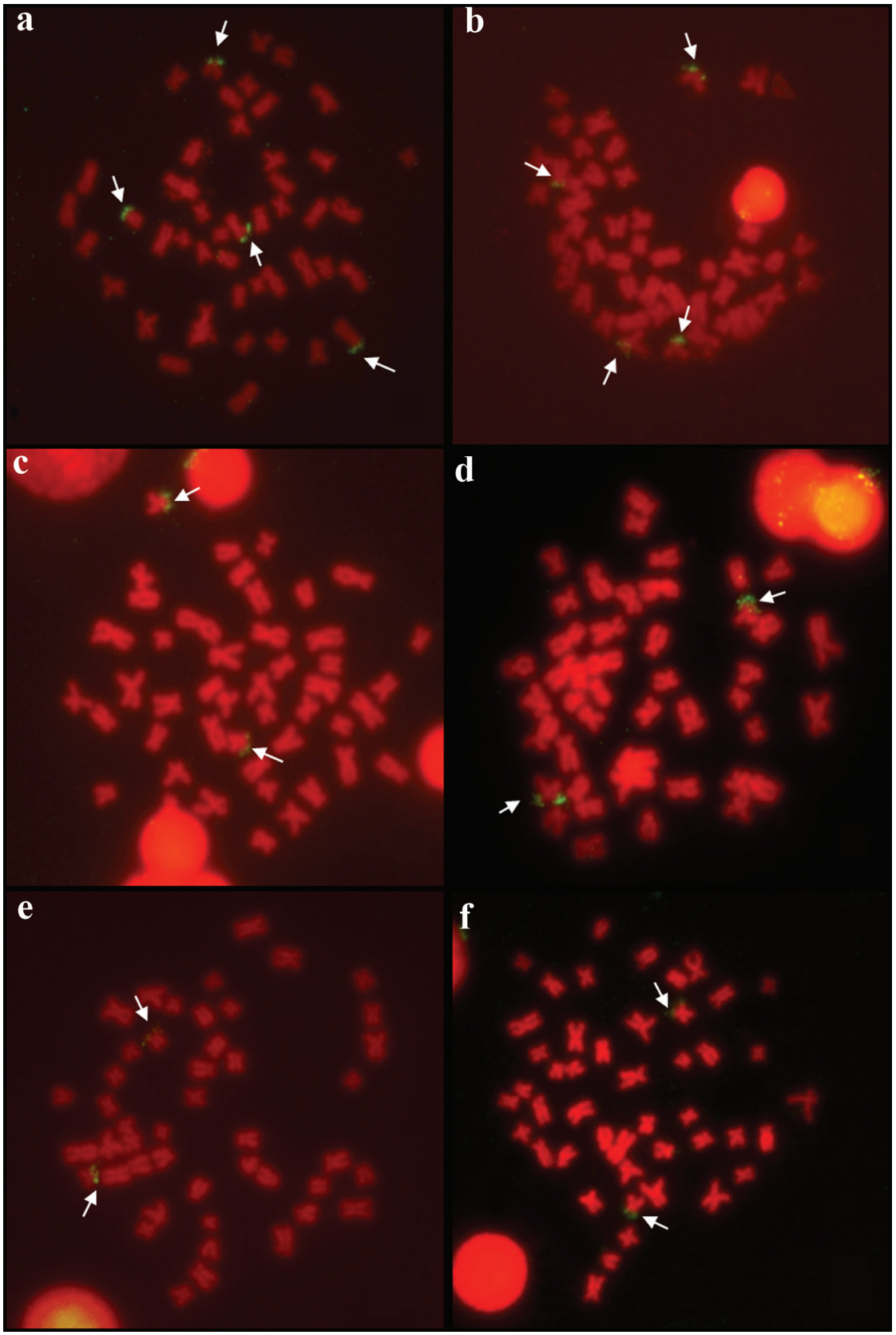

Fig. 3. Somatic metaphases after fluorescent *in situ* hybridization (FISH) with 5s rDNA probes. The arrows indicate the fluorescent marks. **a** = *Pimelodella* sp. (Botucatu, SP); **b** = *Pimelodella spelaea* (São Domingos, GO); **c** = *Pimelodella laurenti* (Cordisburgo, MG); **d** = *Pimelodella* sp. (Colina, SP); **e** = *Pimelodella* sp. (Guapiara, SP); **f** = *Pimelodella* sp. (Pirassununga, SP).

Table 2. Composed haplotypes observed in each population and their absolute and relative frequencies. The order sequence of the used enzymes are *Alu* I, *Hae* III, *Hha* I, *Hinf* I, *Hpa* II, and *Rsa* I.

Population	Haplotype	Absolute Frequency	Relative Frequency
Pimelodella sp. (Araras, SP)	A A A B B B	31	93.93%
	A A A B B D	2	6.06%
Pimelodella sp.(Botucatu, SP)	A A A B A C	23	100.00%
Pimelodella spelaea (São Domingos, GO)	A A A A A A	5	100.00%
Pimelodella laurenti (Cordisburgo, MG)	A A A B A A	4	40.00%
	A B A B A A	6	60.00%
Pimelodella sp. (Colina, SP)	A A A C B B	2	100.00%
Pimelodella sp.(Guapiara, SP)	A C B D A E	4	100.00%
Pimelodella sp. (Pirassununga, SP)	A A A B A C	3	17.64%
	A A A B A A	1	5.88%
	A A A B B B	13	76.47%

Mogi Guaçu River (upper Paraná River) revealed a rare case of NOR polymorphism, where the number of NORs in metacentric and submetacentric chromosomes varied among specimens studied (Dias & Foresti, 1993). The presence of a single NOR is the most common condition among the Siluriformes, specially for groups that present high chromosomal plasticity as Pimelodidae and Heptapteridae (Swarça *et al.*, 2000)

According to Schmid (1980), the correspondence between fluorescent signals after CMA_3 staining and NORs is probably because of a high GC content interspersed with ribosomal genes or between adjacent repetitive DNA sequences. So far, there are no reports about GC rich regions equivalent to heterochromatin segments besides NORs in *Pimelodella* as detected for populations of Guapiara, SP in the present study. This difference in GC content among heterochromatin blocks reveals a differentiation in base richness in these regions, which can be used to identify the studied populations, as previously observed in *Pimelodus* (Garcia & Moreira-Filho, 2005).

Hybridization with 18S rDNA probes provided similar evidence to both silver nitrate and CMA_3 staining, thus confirming the presence of a single NOR system in the studied species. The size heteromorphism observed in this region is often reported in this genus and seems to be derived from unequal chances between homologous during crossing over. Vidotto *et al* (2004) and Swarça *et al*. (2003) used 18S rDNA probes and reported similar results to those from the present study in population studies of *Pimelodella meeki* and in one population of *Pimelodella* aff. *avanhandavae*, respectively.

The C banding technique showed small amounts of constitutive heterochromatin in the analyzed species, with marks restricted to pericentromeric position of some chromosomes and this result are comparable to those obtained by other authors (Swarça *et al*., 2003; Vidotto *et al*., 2004; Vissoto *et al*., 1999). Metacentric chromosomes with heterochromatic blocks on both arms were observed in several species of Pimelodidae and Heptapteridae (Vasconcelos & Martins Santos, 2000; Garcia & Moreira Filho, 2005; Garcia *et al*., 2010) and might be considered a

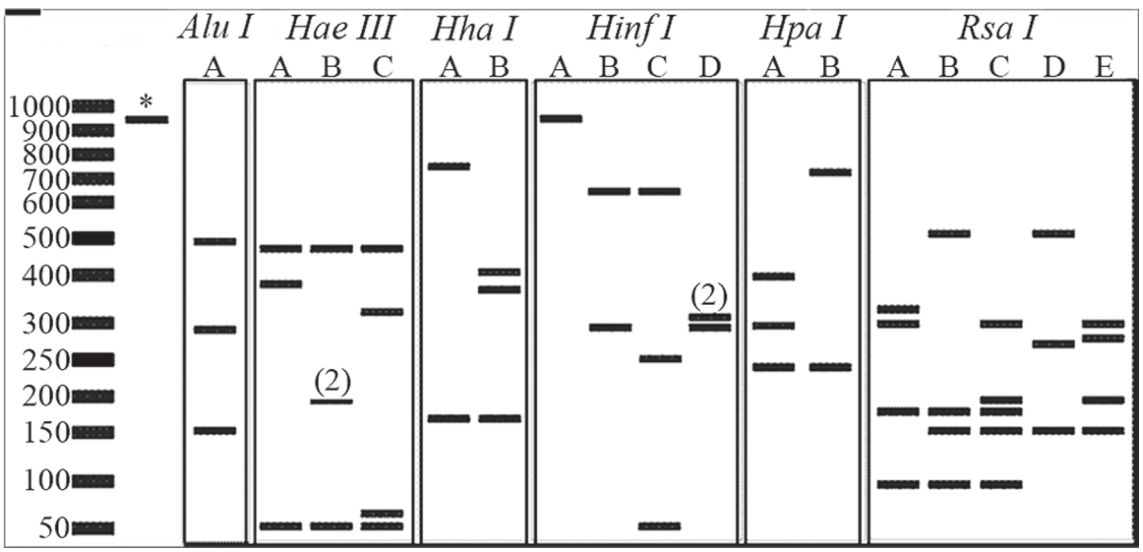

Fig. 4. Schematic representation of approximate sizes of restriction fragments after digestion by endonucleases of mitochondrial ATPase 6 and 8 genes. The letters indicate the haplotyes observed in order of appearance. * = amplified fragment. Ladder = 50pb DNA ladder (Fermentas).

Table 3. Nucleotide diversity (above diagonal) and divergence (below diagonal) among populations.

Populações	*Pimelodella* sp. (Araras, SP)	*Pimelodella* sp. (Botucatu, SP)	*P. spelaea* (São Domingos, GO)	*P. laurenti* (Cordisburgo, MG)	*Pimelodella* sp. (Colina, SP)	*Pimelodella* sp. (Guapiara, SP)	*Pimelodella* sp. (Pirassununga, SP)
Pimelodella sp. (Araras, SP)		0.0212	0.0770	0.0346	0.0150	0.0773	0.0057
Pimelodella sp. (Botucatu, SP)	0.0205		0.0550	0.0148	0.0329	0.0405	0.0160
P. spelaea (São Domingos, GO)	0.0764	0.0550		0.0519	0.0880	0.1018	0.0704
P. laurenti (Cordisburgo, MG)	0.0316	0.0124	0.0495		0.0457	0.0578	0.0285
Pimelodella sp. (Colina, SP)	0.0144	0.0329	0.0880	0.0433		0.1435	0.0191
Pimelodella sp. (Guapiara, SP)	0.0767	0.0405	0.1018	0.0554	0.1435		0.0703
Pimelodella sp. (Pirassununga, SP)	0.0009	0.0118	0.0662	0.0219	0.0148	0.0661	

shared condition of these groups. However this condition may not consist on an useful cytogenetic marker once it could not be used to differentiating species.

The application of 5S rDNA probes allowed the detection of variation in number (2 or 4 sites) of these sequences among studied species, but the obtained data are insufficient to detect any evolutionary pathway about this character in the genus. Therefore, it was not possible to propose the putative occurrence of neither duplication or reduction of these sites. Actually, there are few reports about 5S rDNA FISH studies in Siluriformes. Garcia & Almeida-Toledo (2010) applied this technique to the study of five species of *Pimelodella* and observed the same variation in both number (2, 3 or 4 sites) and location (terminal or pericentromeric) of 5S rDNA signals, just like detected in the present work. Although it was not possible to identified an evolutionary pathway for 5S rDNA sites in *Pimelodella* the number of sites and its positions allowed the differentiation among populations/species that presented the same karyotipic formula, as can be seen for *Pimelodella* sp. (Botucatu, SP x Colina, SP) an *P. speleae* and *Pimelodella* sp. from Guapiara, showing the potential of 5S rDNA as a chromosomal marker.

Among the Siluriformes, the application of techniques

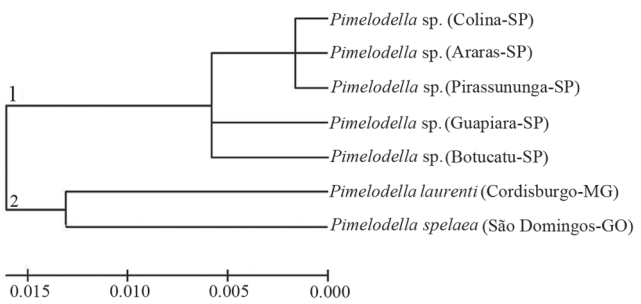

Fig. 5. Phenogram of studied populations. The numbers (1) and (2) are arbitrary values used just for identification of the cited branches, being unrelated to synapomorphies or analyses indexes.

of PCR-RFLP has been very useful in the characterization of species and their hybrids, as in the case of studies involving species of the genus *Silurus* (Triantafyllidis *et al*., 1999) and *Clarias* (Mohindra *et al.*, 2007), in which different species are separates into distinct clades and their populations have some specific haplotypes that enable their identification.

The PCR-RFLP data herein obtained allowed us to detect haplotypic patterns in the studied species (Table 2). Based on haplotype profiles obtained by PCR-RFLP, a phenogram was built to establish the similarities among the studied *Pimelodella* species (Fig. 5). Two branches can be observed in this phenogram: the branch number 1 was composed by the populations from Araras, SP, Colina, SP and Pirassununga, SP and these populations were more closely related with Guapiara, SP and Botucatu, SP. All populations are from the same hydrographic basin, upper Paraná River, but each one was collected in a specific sub basin (Table 1). The populations from Araras, SP, Pirassununga, SP and Colina, SP were more related to each other, probably due to the fact that they belong to the sub basins located geographically closer, revealing a close relationship among them in accordance with the geologic history of their original basins. Haplotypes were different among these populations, but it is noteworthy that 93.93% of Araras, SP specimens and 76.47% of Pirassununga, SP samples shared the haplotype (AAABBB), whereasthe studied samples from Colina, SP presented the haplotype (AAACBB), being differentiated only by the digestion of enzyme Hinf I. The population of the Colina, SP presented an additional restriction site in relation to both Araras, SP and Pirassununga, SP, suggesting that either this site was lost in both or acquired in Colina, SP.

Cave species, *Pimelodella spelaea* and *Pimelodella laurenti,* formed a separate branch (branch number 2), being clustered apart from the other populations probably because they were collected in distinct hydrographic basins lacking connectivity with other sub basins of São Paulo. Such geographic distance leads to isolation in relation to the species

from São Paulo State and it might have been responsible for the present grouping pattern. The similarity between *Pimelodella spelaea* and *Pimelodella laurenti* could be explained by headwater capture events between tributaries from the upper Tocantins River and the middle São Francisco basins, leading to their clustering in the phenogram - to test this hypothesis, studies on additional species from the Tocantins and São Francisco basins are needed in order to apply the comparative method. The headwater event has been recently reported. Ribeiro (2006) described this event in some basins (such as Taubaté, São Paulo, Curitiba, Volta Redonda, among others) and the fish fossils Tremembé Formation (Eocene Oligocene of Taubaté Basin) exemplify this process.

The data obtained with the chromosomal analysis were not directly correlated with the PCR-RFLP analysis in this study. Species sharing the same karyotype formulae were not clustered together and it is likely to be correlated to the fact that PCR-RFLP markers represent the evolution of mitochondrial DNA while karyotype data are related to nuclear genome. Both genomes are characterized by differentiated evolutionary rates and modes, inasmuch as the karyotypic changes might not reflect alterations in mtDNA and *vice versa*. Considering that only presence and absence of restriction sites within the selected mitochondrial gene, the actual genetic variation can be underestimated in the present work. Although no agreement has occurred between these data, this study was very important to genetically analyze samples of the genus *Pimelodella* whose studies are still scarce.

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