

## Cytogenetic characterization of *Hypostomus nigromaculatus* (Siluriformes: Loricariidae)

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*Hypostomus* is the most speciose genus in the family Loricariidae, with approximately 120 species. These fish show a wide morphological and color variation, which hinders the identification of species, mainly of widely distributed representatives. The aim of this study was to contribute to the current knowledge on cytogenetic features of *Hypostomus nigromaculatus*. Three specimens of *H. nigromaculatus*, collected in two tributaries of rio Tibagi, Paraná, and in Cachoeira de Emas, rio Mogi-Guaçu, São Paulo, the latter being the type locality of *H. nigromaculatus*, were studied. Chromosomal preparations were submitted to Giemsa staining, silver nitrate impregnation, C-banding and CMA<sub>3</sub> and DAPI fluorochromes staining. All samples presented  $2n = 76$ , but the rio Mogi-Guaçu sample differed from those from tributaries of rio Tibagi in relation to karyotype formulae, distribution and composition of heterochromatin, and NOR location. The silver nitrate staining revealed the presence of multiple Ag-NORs for all samples, but with differences on the location on chromosomes. CMA<sub>3</sub> staining revealed bright signals equivalent to NOR-bearing chromosomal segments; such sites were characterized by negative, i.e. unstained, marks after DAPI staining. The pattern of heterochromatin distribution was distinctive among samples from rio Mogi-Guaçu and tributaries of rio Tibagi. The differences observed between the sample from rio Mogi-Guaçu and the ones from tributaries of rio Tibagi allow us to suggest that these samples are presently isolated. Further analyses are necessary to ascertain whether such isolation refers to distinct populations or characterizes true different species.

O gênero *Hypostomus* é um dos mais especiosos na família Loricariidae, tendo aproximadamente 120 espécies. Apresenta uma ampla diversidade quanto ao padrão de coloração e morfologia, o que dificulta a identificação de determinadas espécies, principalmente aquelas com ampla distribuição geográfica. Para isso os dados obtidos neste trabalho contribuem para os estudos citogenéticos de *Hypostomus nigromaculatus*. Foram analisados três exemplares de *H. nigromaculatus* de afluentes do rio Tibagi, Paraná e Cachoeira de Emas, rio Mogi-Guaçu, São Paulo, sendo esta última, a localidade tipo de *H. nigromaculatus*. Os cromossomos foram submetidos à coloração convencional (Giemsa), impregnação por nitrato de prata, bandamento-C e coloração com os fluorocromos CMA<sub>3</sub> e DAPI. Todos os exemplares apresentaram  $2n = 76$ , no entanto com diferença quanto às fórmulas cariotípicas, distribuição e composição da heterocromatina. O nitrato de prata detectou RONS múltiplas para as amostras, porém com diferenças quanto à localização nos cromossomos. A coloração com fluorocromo CMA<sub>3</sub> foi correspondente aos cromossomos Ag-RONS, na coloração com DAPI foram observadas bandas negativas, ou seja, não coradas. O padrão de distribuição da heterocromatina foi diferente para as amostras do rio Mogi-Guaçu e dos tributários do rio Tibagi. As diferenças observadas entre as amostras de rio Mogi-Guaçu e afluentes do rio Tibagi permitem-nos sugerir que essas amostras estejam atualmente isoladas. Outras análises são necessárias para determinar se as amostras analisadas são populações distintas ou trata-se de espécies diferentes.

**Key words:** karyotype evolution, Nucleolus organizer region, Heterochromatin, Hypostominae.

### Introduction

*Hypostomus* is one of the most speciose genera in the subfamily Hypostominae, with approximately 120 nominal species (Weber, 2003). These fish occur almost everywhere in the East Andean tropical and temperate South America, inhabiting an array of aquatic habitats, although being usually found in running water systems (Montoya Burgos, 2003).

Systematists have found it very difficult to identify species in this group, due to the large number of species belonging to the genus and due to their large intra-specific variation of both morphology and color pattern (Reis *et al.*, 1990). Due to this large variation, molecular studies using allozymes and mtDNA have been carried out to help species identification and determine the phylogenetic relationships of some species within the genus (Zawadzki *et al.*, 2001, 2005).

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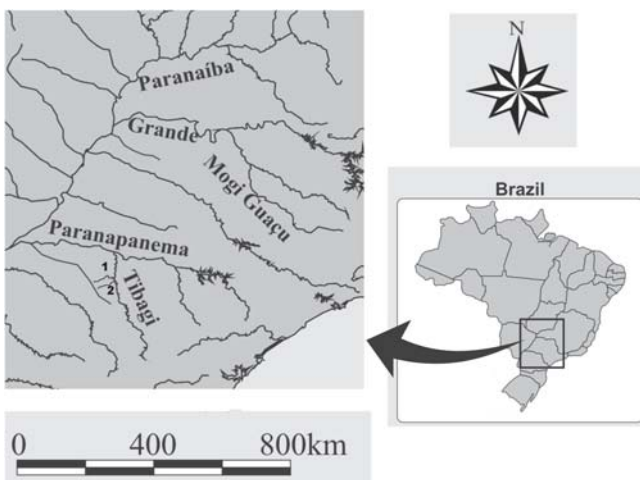
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In relation to the diploid number, karyotype macrostructure and chromosomal banding, this group can be considered non-conservative (Artoni & Bertollo, 1996; Alves *et al.*, 2003). Chromosomal rearrangements have played a major role in the karyotype evolution of Hypostominae (Artoni & Bertollo, 1996), including a case of polymorphism related to the presence of supernumerary chromosomes (Scavone & Júlio Jr., 1994). To date, most of cytogenetic data about the genus *Hypostomus* are restricted to diploid number, karyotype formulae and NOR location. The diploid number range from 52 in *H. emarginatus* (Artoni & Bertollo, 2001) to 84 in *Hypostomus* sp. 2- rio Perdido NUP 4249 (S. S. Cereali, pers. commun.). The number of NOR-bearing chromosomes varies from 2 to 8 chromosomes, usually carrying terminal rDNA sites on short or long arms (Artoni & Bertollo, 2001; Alves *et al.*, 2005).

In the present study, specimens of *Hypostomus nigromaculatus* (Schubart, 1964) from rio Mogi-Guaçu, Cachoeira de Emas, the type locality of this species, and from two other localities in the rio Tibagi, were cytogenetically examined in order to characterize a possible geographic variation in this species. Furthermore, these represent the first cytogenetic data on *H. nigromaculatus*.

### Material and Methods

*Hypostomus nigromaculatus* is widespread throughout Middle and Upper Paraná river basin in South America, being Cachoeira de Emas, rio Mogi-Guaçu, a tributary of rio Grande, in the city of Pirassununga, state of São Paulo, its type locality. Fifteen specimens of *Hypostomus nigromaculatus* were collected at three different localities: Cachoeira de Emas – rio Mogi-Guaçu, (NUP 5071, three males and four females), in Pirassununga, São Paulo, Brazil; ribeirão Três Bocas (NUP 5070, two males and three females); and ribeirão dos Apertados (MZUEL 4705, three females). The last two sites



**Fig.1.** Hydrographic map showing rio Mogi-Guaçu, rio Tibagi, 1 = ribeirão Três Bocas and 2 = ribeirão dos Apertados (tributaries of the rio Tibagi).

are tributaries of the rio Tibagi, located in the municipality of Londrina, Paraná, Brazil (Fig.1). NUP refers to the ichthyological collection of NUPELIA (NUP: Núcleo de Pesquisas em Limnologia, Ictiologia e Aqüicultura, da Universidade Estadual de Maringá, Brazil) and MZUEL refers to the ichthyological collection of Museu de Zoologia da Universidade Estadual de Londrina, Brazil.

Mitotic chromosomes were obtained according to the conventional technique of Bertollo *et al.* (1978). The chromosomes were classified into three groups: metacentric (m), submetacentric (sm) and subtelocentric-acrocentric (st-a), according to Levan *et al.* (1964). Nucleolar organizer regions were detected by silver nitrate staining (Ag-NORs) (Howell & Black, 1980). GC- and AT-rich sites were stained with Chromomycin A<sub>3</sub> (CMA<sub>3</sub>) and 4' 6-diamidin-2-phenylindole (DAPI) (Schweizer, 1976; Schmid, 1980; Christian *et al.*, 1998). C-banding was performed to determine the pattern of heterochromatin distribution (Sumner, 1972) and chromosomes were stained with Giemsa and with the fluorochromes CMA<sub>3</sub> and DAPI in order to verify their compositional nature.

### Results

The seven specimens from rio Mogi-Guaçu showed a diploid number of  $2n=76$  and a karyotype formulae of  $8m+20sm+48st-a$ , with a fundamental number (FN) of 104 (Fig. 2a). Four specimens presented a secondary constriction on long arms of up to two non-homologous and large st-a chromosomes (25<sup>th</sup> and 28<sup>th</sup>) (Fig. 3a-I).

All specimens from both samples in tributaries of rio Tibagi presented a diploid number of  $2n=76$ , with a karyotype formulae of  $6m+20sm+50st-a$  and a FN equal to 102 (Fig. 2b). Three specimens showed up to three chromosomes bearing secondary constrictions on short arms of a large st-a pair (16<sup>th</sup>) and on long arms of another st-a chromosome (27<sup>th</sup>). Pictures related to the sample from Apertados are not shown in the figures, once the results were identical to those from ribeirão Três Bocas (Fig. 3b-I).

In the sample from rio Mogi-Guaçu, silver nitrate staining of the chromosomes revealed the presence of multiple Ag-NORs in two or three large st-a chromosomes, located at terminal position on long arms, corresponding to pairs 25 and 28. On the pair 25, positive staining was observed in one homologous, and a size heteromorphism was detected in the pair 28 of four specimens, in agreement with the secondary constrictions, with distinct individual pattern within this sample (Fig. 3a-II).

Silver nitrate staining of the chromosomes in specimens from tributaries of the rio Tibagi showed until three stained chromosomes with marks located at terminal region on long arms of one st-a chromosome, corresponding to an element from the pair 27, and at terminal region on short arms of a st-a pair (pair 16), also characterized by size heteromorphism in three specimens (Fig. 3b-II).

Staining with the fluorochrome CMA<sub>3</sub> detected until three positively stained sites on Ag-NOR-bearing chromosomes in

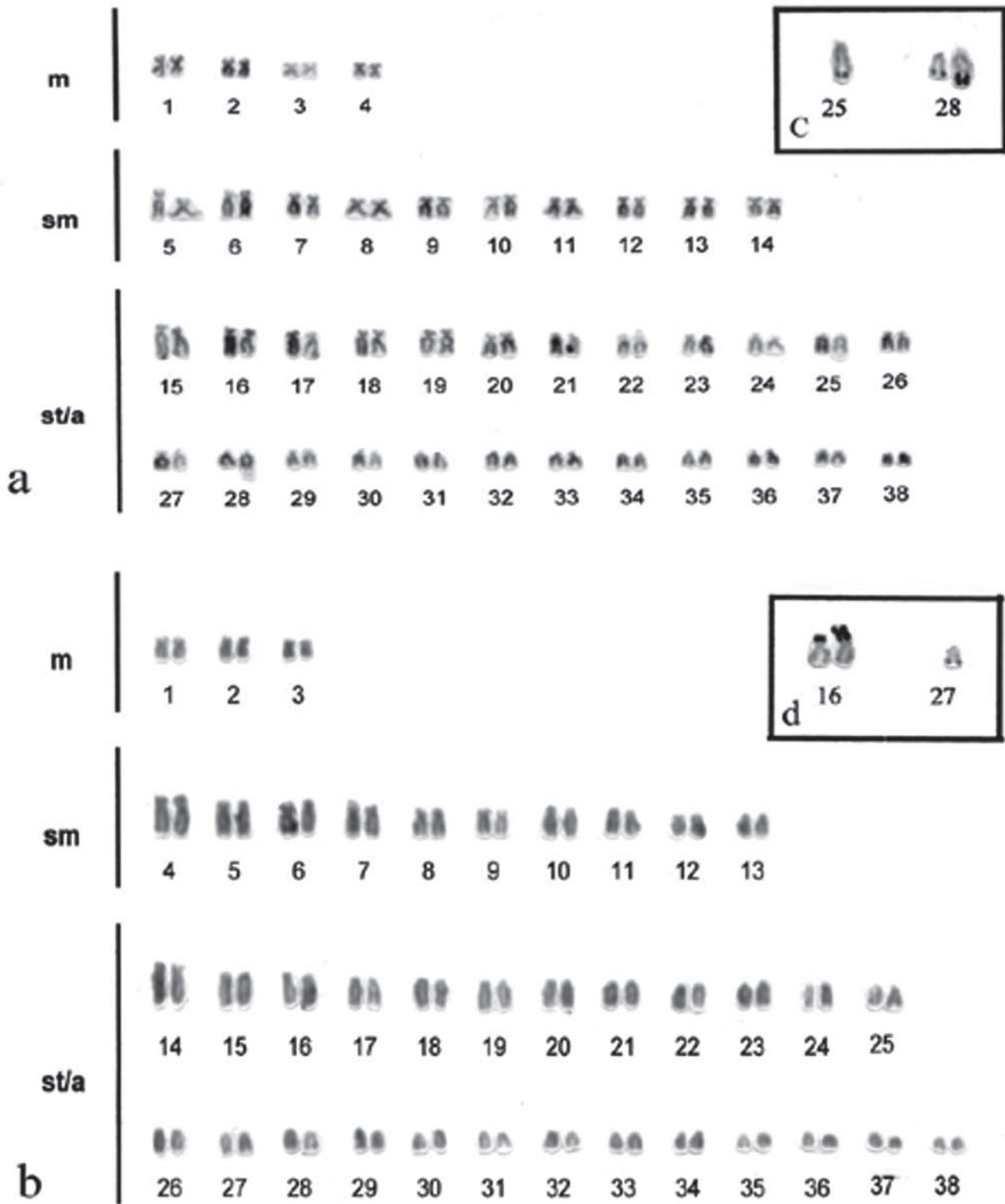


Fig. 2. Karyotypes of *Hypostomus nigromaculatus* with conventional Giemsa staining: a) rio Mogi-Guaçu and b) tributaries of the rio Tibagi. Ag-NOR-bearing chromosomes from rio Mogi-Guaçu (c) and tributaries of the rio Tibagi (d) are framed.

the rio Mogi-Guaçu sample, which also showed a size heteromorphism between homologous. Besides, negatively stained sites (CMA<sub>3</sub>) were observed close to NORs (Fig. 4a). DAPI staining showed negative regions (DAPI-) corresponding to NORs while the chromosomal segment close to NORs (CMA<sub>3</sub>) was stained with DAPI (Fig. 4b).

All individuals analyzed from the tributaries of the rio Tibagi showed positive CMA<sub>3</sub> staining coincident with Ag-NOR sites and also at the centromeric region of some metacentric chromosomes (Fig. 4c). DAPI regions were observed corresponding to Ag-NORs in both samples from rio Tibagi basin (Fig. 4d).

Heterochromatin, detected by C-banding, was distributed at pericentromeric region of a metacentric pair and as large terminal blocks on long arms of two pairs (st-a), with heterochromatic segments adjacent to secondary constrictions in all individuals from rio Mogi-Guaçu sample (Fig. 5a). The same pattern was observed with DAPI- and CMA<sub>3</sub>-stained C-banding, with presence of fluorescent bands at terminal position on long arms of st-a chromosomes, although Ag-NORs could not be distinguished from heterochromatin (Fig. 5b and c).

C-banded chromosomal preparations of individuals from both tributaries of rio Tibagi revealed heterochromatin blocks at terminal region on long arms of two st-a pairs, at centromeric region of several chromosomes and associated with Ag-NORs. The Ag-NOR-bearing chromosomal pair also showed size heteromorphism between homologous (Fig. 5d). C-banding stained with CMA<sub>3</sub> presented fluorescent marks at terminal region of some st-a chromosomes, either on short or long arms, including Ag-NOR chromosomes (Fig. 5e). C-banding with DAPI staining showed positive bands at centromeric region of two metacentric pairs and some negative sites corresponding to NORs (Fig. 5f).

## Discussion

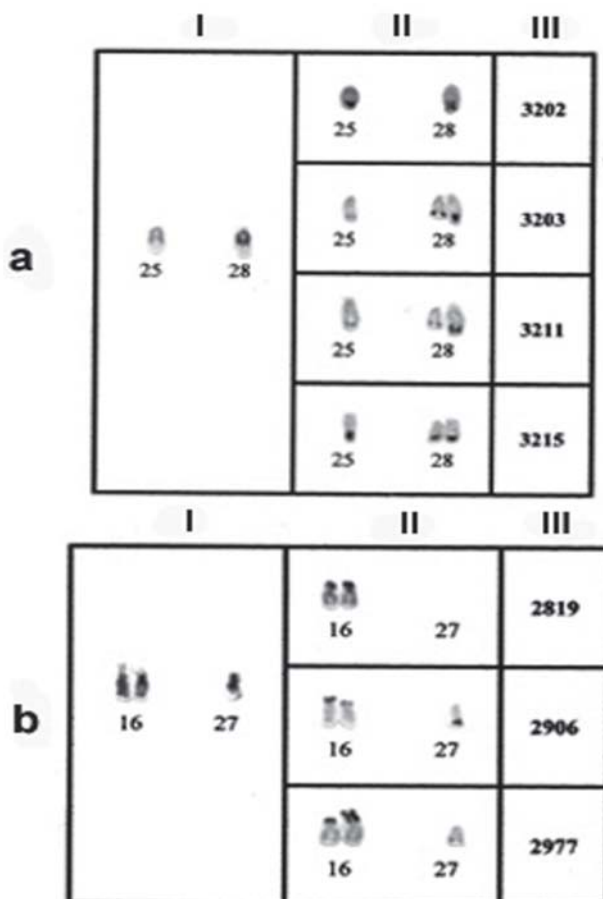
Conventional cytogenetic analysis of the three samples of *H. nigromaculatus* showed a same diploid number; but the specimens collected in the tributaries of rio Tibagi differed from those from rio Mogi-Guaçu in relation to FN and location of secondary constriction and Ag-NOR-associated heterochromatin. Changes in the karyotype formulae without modifications in the diploid number are usually determined by structural chromosomal rearrangements, such as pericentric inversions, along the karyotype evolutionary pathways of the group.

After silver nitrate staining, the chromosomes all individuals analyzed showed multiple NORs and a size heteromorphism between homologous, with distinct locations according to each sample. The specimens from rio Mogi-Guaçu presented terminal Ag-NORs on the long arms of three large st-a chromosomes; whereas the specimens from tributaries of the rio Tibagi presented Ag-NORs on the short arms of a large st-a pair and long arms of a small st-a chromosome, also terminally located.

As for the size heteromorphism of Ag-NOR sites in the samples from tributaries of the rio Tibagi, it is not known whether this feature is related to ribosomal cistrons or to heterochromatin amplification, since both are associated. Such Ag-NOR size polymorphism can be a consequence of unequal crossovers. This feature has been widely reported in other groups of Neotropical fish (Foresti *et al.*, 1981; Almeida-Toledo *et al.*, 2000) and it is also present within Loricariidae, such as Hypoptopomatinae (Andreato *et al.*, 1994; Alves *et al.*, 2003, 2006), Hypostominae (Artoni & Bertollo, 1996) and Loricariinae (Giuliano-Caetano, 1998).

CMA<sub>3</sub> staining detected sites corresponding to Ag-NORs, thus demonstrating that ribosomal sites in the three samples studied are GC-rich, as corroborated by the presence of DAPI negative bands.

The distribution of heterochromatin and its composition, determined by CMA<sub>3</sub> and DAPI staining, also differed among samples. The rio Mogi-Guaçu sample showed both AT- and GC-rich heterochromatin segments, with large terminal blocks in two st-a pairs, including one NOR-bearing chromosomal pair. However, the sample from tributaries of the rio Tibagi



**Fig. 3.** Chromosomes bearing of *Hypostomus nigromaculatus* secondary constriction: a= sample from rio Mogi-Guaçu and b= sample from tributaries of the rio Tibagi; I- Giemsa-stained; II- silver nitrate staining; III- number of specimens examined.



showed a higher amount of heterochromatin widely distributed at centromeric and telomeric regions, and AgNORs. The heterochromatin associated with NORs was positively stained by CMA<sub>3</sub> while DAPI stained centromeric region of two meta-centric pairs.

According to Oliveira *et al.* (1988) and Almeida-Toledo *et al.* (1993), the migratory rate and population density can influence the karyotypic macrostructure of a group. That is, the greater the mobility and number of individuals of a group, the more stable the karyotypic macrostructure should be, since gene flow is higher and the fixation of some chromosomal alteration would be hindered. Most species of the genus *Hypostomus* are non-migratory and generally comprise small populations (Britski *et al.*, 1984). Therefore, the differences observed among *H. nigromaculatus* samples in this study might be related to their populational structure.

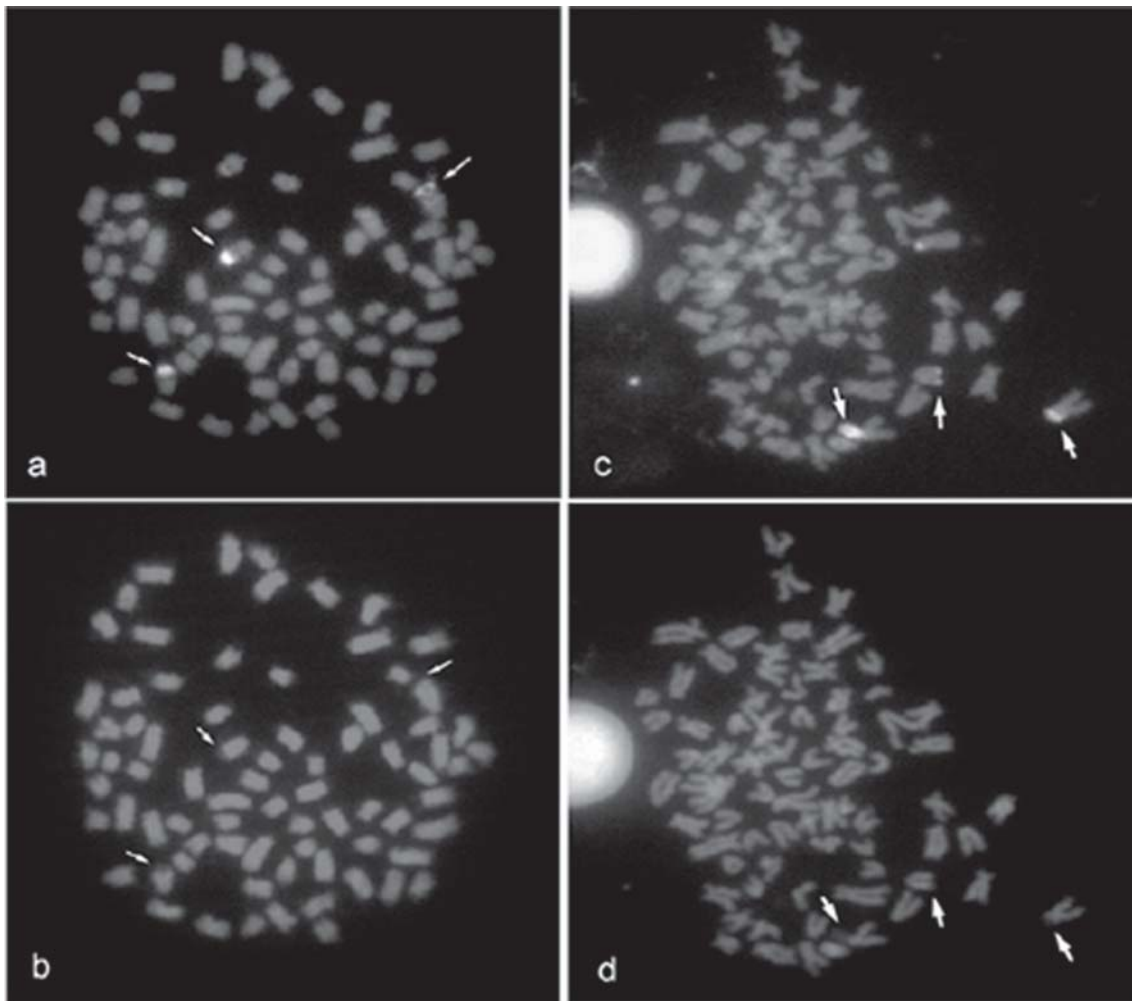
The sample from rio Mogi-Guaçu has been geographically isolated, for a long time, from the tributaries of the rio Tibagi. Thus, the lack of gene flow between them would favor the fixation of distinctive modifications in both micro- and

macrostructure in each sample, putatively resulting in an incipient speciation process.

Analogously, the karyotype similarity observed between samples from different tributaries of the rio Tibagi is likely related to the fact that both streams belong to the Tibagi river basin and they are geographically closer to each other.

Based on the results obtained in this work, samples from rio Tibagi basin and rio Mogi-Guaçu show considerable cytogenetic differences (FN, Ag-NOR location, and composition and distribution of heterochromatin). These differences corroborate previous reports, showing that this fish group is characterized by non-conserved features in relation to both chromosomal micro- and macrostructure (Michelle *et al.*, 1977; Artoni & Bertollo, 1996).

Nevertheless, regarding to external morphology, no apparent differences among the specimens from the three samples studied were observed, or these differences could be very subtle. Therefore, the samples studied could represent either distinct species but morphologically similar (cryptic species), or a single species, morphologically identical,

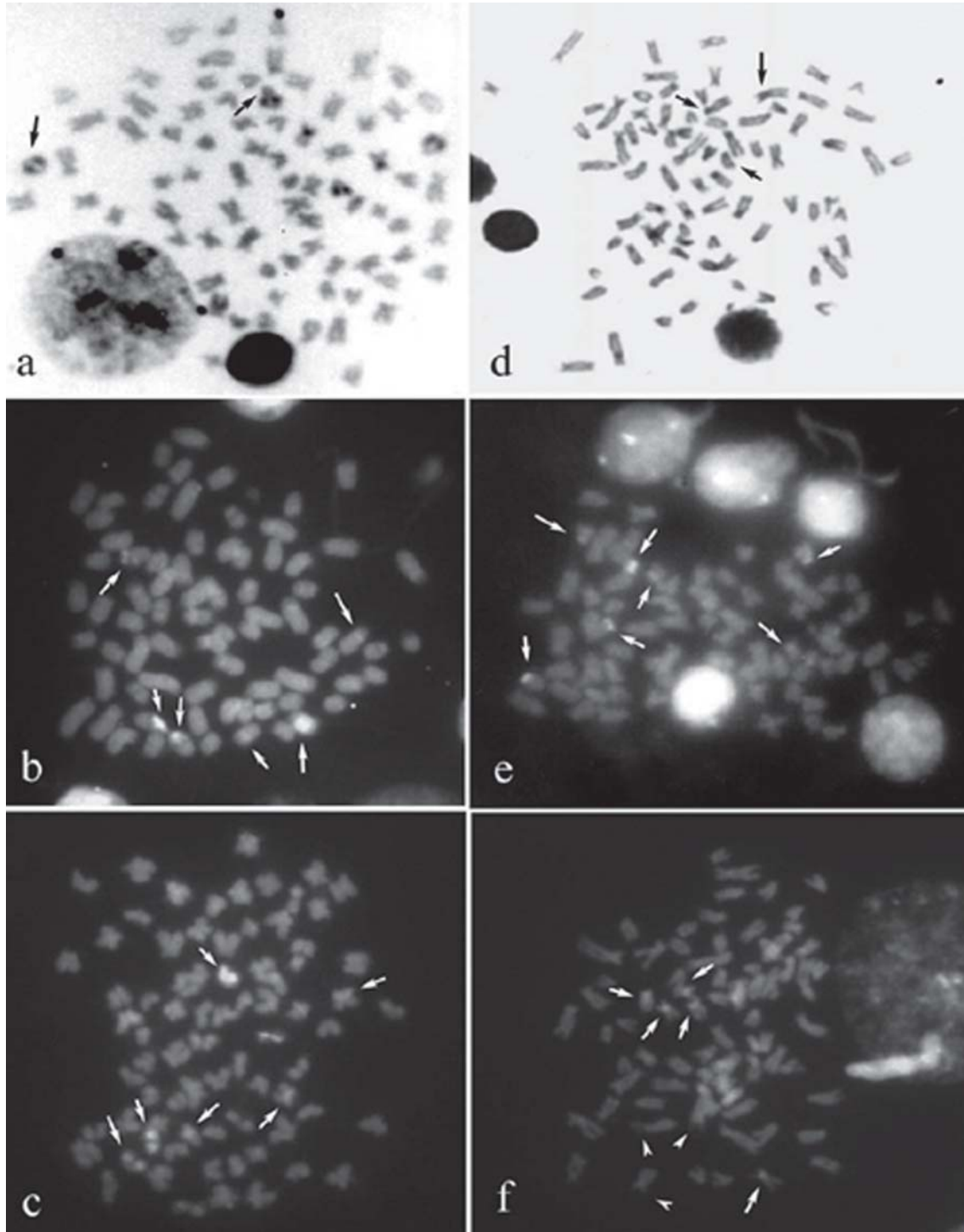


**Fig. 4.** Metaphases of *Hypostomus nigromaculatus* stained with CMA<sub>3</sub> and DAPI, respectively. **a)** and **b)**: Mogi-Guaçu; **c)** and **d)**: tributaries of the rio Tibagi. The arrows indicate the NORs.

but showing a considerable intra-specific polymorphism. Cytogenetic studies focusing the population structure of *Hypostomus* species are not available so far. However, Zawadzki *et al.* (2002) analyzed two samples of *H. margaritifer*, one from the Itaipu reservoir and another from rio Corumbá, a tributary of rio Paranaíba using allozymes.

The authors found no diagnostic character but differences in the expected heterozygosity between populations, what they assumed to be due to a non-random mating system.

The genetic analysis of specimens from the type locality of those species known to be polymorphic can be a powerful tool to the taxonomic study of Neotropical fish. Such ap-



**Fig. 5.** C-banded metaphases of *Hypostomus nigromaculatus* stained with Giemsa, CMA<sub>3</sub> and DAPI, respectively. **a), b)** and **c)**: rio Mogi-Guaçu; **d), e)** and **f)**: tributaries of the rio Tibagi. The black arrows indicate the NORs, the white arrows indicate heterochromatic blocks and the arrowheads indicate the negative bands.

proach links cytogenetic, allozyme or molecular patterns to a specific taxon. When a population previously thought to belong to that taxon diverges considerably from the type locality, it should be taxonomically examined. Then, new questions can be raised, not only from an evolutionary genetic point of view, but also related to biogeographic and taxonomic issues.

In the present report, it should be pointed out that rio Mogi-Guaçu makes part of rio Grande basin and, just like rio Paranaíba, it presents a relatively differentiated ichthyofauna from the lower portion of the Upper Paraná river basin. Species such as *Hypostomus fluviatilis* (Schubart, 1964) and a new species of *Hypostomus* (C.H. Zawadzki and F. Langeani, pers. com.) from rio Grande, the characid *Oligosarcus planaltinae* (Menezes & Géry, 1983), as well as the curimatid *Steindachnerina corumbae* (Pavanelli & Britski, 1999), from the Paranaíba basin, are apparently limited to Grande-Paranaíba basin. On the other hand, the Tibagi basin also presents a highly endemic fish fauna, as demonstrated by the recent description of two new loricariid species of the genus *Isbrueckerichthys* (Jerep *et al.*, 2006) in this region. Consequently, small populations of species and genera known to be polymorphic are expected to show some degree of genetic or morphological differentiation.

The cytogenetic differences among the samples analyzed here are remarkable but caution needs to be taken prior to determine whether these samples actually belong to the same species or not, since further additional studies on *Hypostomus nigromaculatus* are still required.

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