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Research Article

# Cytogenetic markers as diagnoses in the identification of the hybrid between Piauçu (*Leporinus macrocephalus*) and Piapara (*Leporinus elongatus*)

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## Abstract

The genetic monitoring of interspecific hybrids involves the application of methodologies able to provide an easy and indubitable genetic characterization of both parental and hybrid individuals. In the present work, cytogenetic techniques were used to identify a hybrid lineage of "Piaupara" in order to caracterize them in relation to the parental species, *Leporinus macrocephalus* (piauçu) and *L. elongatus* (piapara). The cytogenetic analysis revealed that *L. macrocephalus* presented 2n = 54 chromosomes and a nucleolar organizer regions (NOR) at the telomere of the long arm of the submetacentric chromosome pair 2. Analysis of constitutive heterochromatin (C-banding) revealed a conspicuous block at the pericentromeric region on the long arm of a submetacentric chromosome pair. *L. elongatus* presented the same diploid number, 2n = 54, and a karyotypic formula similar to that of *L. macrocephalus*. The NORs were also at the telomere of the long arm of the submetacentric pair 2, which was morphologically different from that of *L. macrocephalus*. Heterochromatic blocks were observed at both telomeres of a submetacentric chromosome pair. The hybrid "Piaupara" presented the same diploid number (2n = 54) and karyotypic formula as the parental species and there were no visible differences between parental and hybrid individuals. Differently from the Giemsa staining, NOR- and C-banding analysis showed marked differences which allowed the identification of the hybrids by the different morphology and/or size of the chromosomes carrying the NORs and patterns of heterochromatin distribution in their chromosomes. Such genetic studies are important for fish culture since they can provide tools for monitoring natural and artificial hybridization. They are also useful in biological conservation programmes and in the proper management of natural and reared fish stocks.

*Key words:* interspecific hybrid, fish culture, fish cytogenetics.

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## Introduction

Biological sciences and, particularly, biotechnological studies have played a major role on the development of fish culture over the last decades. The improvement of current methodologies and its application in studies of fish biology and genetics are necessary to develop a better genetic management of both captive stocks and natural populations (Porto-Foresti and Foresti, 2004).

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Interspecific hybridization focused on productivity increase and formation of sterile lineages represents one of the main classic methods of genetic manipulation applied in fish farms. Most of natural fish hybrids are found in continental waters, where the frequency of hybridization and speciation is remarkably higher than that found in marine species, in which hybrids are generally rare (Hubbs, 1955).

The use of artificial hybridization in fish was initiated about 30 years ago in Brazil by the Departamento de Obras Contra a Seca (DNOCS) and involved different species of tilapias (Toledo-Filho *et al.*, 1998). Nowadays, it involves a large number of interspecific crosses among Neotropical

fish species (Table 1). In this way, the widespread production of interspecific hybrid fishes justifies their effective characterization and the elaboration of monitoring programmes at the production level.

The expressive results obtained with the use of interspecific hybridization techniques in fish need to be carefully interpreted in face of the potential biological risks that hybrids pose to the environment. If fertile, they can genetically contaminate both natural and reared parental stocks (Ryman and Utter, 1987). Otherwise, in natural habitats, they may compete in different ways with parental lineages (Toledo-Filho *et al.*, 1998). Therefore, the genetic identification, characterization and monitoring of hybrids produced by fish breeding farms may provide important information which could be used in hybridization programmes applied to fish culture.

Currently, interspecific hybrid individuals between the species Piauçu (*Leporinus macrocephalus*) and Piapara (*L. elongatus*) are being produced in Brazilian fish cultures. The parental species belong to the family Anostomidae, which comprises twelve identified genera and represents an important freshwater fish group widespread throughout the Neotropical region (Géry, 1977). The most representative genera of this family are *Leporinus* (87 species) and *Schizodon* (14 species) (Garavello and Britski, 2003), and in the *Leporinus* group many species constitute important fishery resource to specific communities, such as *L. macrocephalus*, *L. elongatus* and *L. obtusidens*.

Although the presence of 54 chromosomes remains constant within the species of the family Anostomidae (Galetti Jr. *et al.*, 1981a), interesting chromosome rearrangements seem to have occurred in this group. Galetti Jr. *et al.* (1991b) reported perceptible C-banding patterns differences in chromosomes of representatives of the family Anostomidae and, furthermore, Galetti Jr. *et al.* (1984) demonstrated that the nucleolus organizer regions (NORs) are located on different chromosomes or at distinct chromosome positions. These characteristics can be used as a tool for unambiguous species identification in this family.

Besides the occurrence of normal homomorphic karyotypes in some *Leporinus* species, the occurrence of a ZZ/ZW sex chromosomes system was also observed (Galetti Jr. *et al.*, 1981b; Galetti Jr. and Foresti, 1986, 1987; Galetti Jr. *et al.*, 1995; Molina and Galetti Jr., 2006). It involved a pair of large meta- and submetacentric chromosomes equivalent in size to the second pair, with the smaller metacentric chromosome corresponding to the Z and the submetacentric chromosome corresponding to the W chromosome (Galetti Jr. *et al.*, 1981b; Galetti Jr. and Foresti, 1987).

As the production of interspecific hybrids is currently a common practice among fish breeders, the major goal of the present work was to characterize and differentiate parental species of *Leporinus* and their artificial interspecific hybrid. We aimed at providing a better understanding on the dynamics of the interspecific hybridization processes in fish, at supporting projects on fish hybridization developed by farmers, and at establishing guidelines for biological conservation programmes involving these species.

## Material and Methods

From the parental lines, 19 specimens of Piauçu (*Leporinus macrocephalus*) and 20 individuals of Piapara (*Leporinus elongatus*) were cytogenetically analyzed.

**Table 1** - A list of fish species and crosses that produce hybrids identified through the parental species.

| Parental generation | | Hybrid |
|---|---|---|
| Parental female | Parental male | |
| Tambaqui - *Colossoma macropomum* | Pacu - *Piaractus mesopotamicus* | "Tambacu" |
| Pacu - *Piaractus mesopotamicus* | Tambaqui - *Colossoma macropomum* | "Paqui" |
| Tambaqui - *Colossoma macropomum* | Pirapitinga - *Piaractus brachypomus* | "Tambatinga" |
| Pirapitinga - *Piaractus brachypomus* | Tambaqui - *Colossoma macropomum* | "Pirambaqui" |
| Pacu - *Piaractus mesopotamicus* | Pirapitinga - *Piaractus brachypomus* | "Patinga" ou "Papi" |
| Pirapitinga - *Piaractus brachypomus* | Pacu - *Piaractus mesopotamicus* | "Pirapicu" |
| Piauçu - *Leporinus macrocephalus* | Piapara - *Leporinus elongatus* | "Piaupara" |
| Piapara - *Leporinus elongatus* | Piauçu - *Leporinus macrocephalus* | "Piapaçu" |
| Pintado - *Pseudoplatystoma corruscans* | Cachara - *Pseudoplatystoma fasciatum* | "Pintachara" |
| Cachara - *Pseudoplatystoma fasciatum* | Pintado - *Pseudoplatystoma corruscans* | "Cachapinta" |
| Pintado - *Pseudoplatystoma corruscans* | Jurupoca - *Hemiosorubim platyrhynchos* | "Pintajuru" |
| Pintado - *Pseudoplatystoma corruscans* | Pirarara - *Phractocephalus hemioliopterus* | "Pintapira" |
| Cachara - *Pseudoplatystoma fasciatum* | Pirarara - *Phractocephalus hemioliopterus* | "Cachapira" |
| Pintado - *Pseudoplatystoma corruscans* | Jandiá - *Leiarius marmoratus* | "Pintadiá" |
| Jandiá - *Leiarius marmoratus* | Pintado - *Pseudoplatystoma corruscans* | "Janditado" |

Crosses performed between these species resulted in the production of the interspecific hybrid "Piaupara" - the lineage obtained by using females of Piauçu and males of Piapara. The cytogenetic analysis in hybrids comprised 21 specimens of "Piaupara". All the specimens analyzed were obtained from the stock belonging to the Kabeya Aquaculture, Penápolis (SP), Brazil and were identified and deposited in the fish collection of the Laboratory of Fish Genetics, UNESP, Bauru (SP), Brazil.

Chromosome preparations were obtained from gill and kidney tissues using the technique described by Foresti *et al.* (1981). Silver staining of the nucleolus organizer regions followed the technique of Howell and Black (1980) and C-banding was performed according to Sumner (1972). Chromosome morphology was determined on the basis of arm ratio as proposed by Levan *et al.* (1964) and the chromosomes were classified as metacentric (M), submetacentric (SM), subtelocentric (ST) and acrocentric (A).

## Results and Discussion

The study of interspecific hybrids depended on the cytogenetic identification of the parental species Piauçu (*Leporinus macrocephalus*) and Piapara (*L. elongatus*), thus chromosome preparations both species were obtained.

### Cytogenetic identification of Piauçu (*L. macrocephalus*) and Piapara (*L. elongatus*)

Nineteen specimens of *L. macrocephalus* (Piauçu) (five females and 14 males) were analyzed. They presented a diploid number of 2n = 54 and their karyotype was similar to that described by Galetti Jr. and Foresti (1987). The fundamental number (FN) in this species was 108 and the chromosome formula comprised meta- and submetacentric chromosomes and a ZZ/ZW sex chromosome pair (Figure 1a).

Twenty specimens of *L. elongatus* (Piapara) (eight females and 12 males) were analyzed. The specimens showed 2n = 54, with a karyotype composed of meta- and submetacentric chromosomes and a fundamental number (FN) equal to 108, confirming previous reports by Galetti Jr. and Foresti (1986), Koehler *et al.* (1997) and Molina *et al.* (1998). This species also presented the ZZ/ZW sex chromosome system (Figure 1b).

According to Vari (1983), the family Anostomidae shares the same phylogenetic unit with Curimatidae, Prochilodontidae and Chilodontidae. Karyotypic studies carried out in representatives of the Anostomidae family showed a basic karyotype composed of 54 mostly meta- and submetacentric chromosomes (Galetti Jr. *et al.*, 1981a), similar to the karyotypes of *L. macrocephalus* and *L. elongatus* herein studied.

Furthermore, the cytogenetic analysis confirmed the occurrence of a chromosomal heteromorphism related to sex in both species (Figures 1a and 1b) previously observed in some *Leporinus* species (Galetti Jr. *et al.*, 1981b; Galetti Jr. and Foresti, 1986, 1987; Galetti Jr. *et al.*, 1995; Molina *et al.*, 1998; Molina and Galetti Jr., 2006).

Analysis in three genera of the family Anostomidae (*Leporinus*, *Leporellus* and *Schizodon*) demonstrated that, superficially, they have a great karyotypic similarity (Galetti Jr. *et al.*, 1981a). Further cytogenetic analysis applying silver nitrate staining of the nucleolus organizer regions (NORs) were informative enough to characterize some species in this group. Although all of them presented a single NOR-bearing pair, the NORs were at different chromosome positions. The differences might be related to chromosome rearrangements as translocations and/or inversions, thus representing cytogenetic markers for these species (Galetti Jr. *et al.*, 1984).

Silver nitrate staining on chromosome preparations of *L. macrocephalus* and *L. elongatus* revealed the presence of a single chromosome pair bearing ribosomal cistrons in both species. The NORs were located at a terminal position on the long arm of a submetacentric chromosome (pair 2) in both species (Figures 1a and 1b - inbox). However, as the NOR-bearing chromosomes in each species have different sizes and/or morphologies, they can be useful cytological markers for the identification of interspecific hybrids between these species.

C-banding of *L. macrocephalus* and *L. elongatus* chromosomes revealed the presence of heterochromatic blocks over centromeric and pericentromeric regions of some chromosomes in both species. In *L. macrocephalus*, conspicuous interstitial blocks were present in the pericentromeric region at the long arms of the submetacentric chromosomes of the third pair (Figure 2a - inbox). In *L. elongatus*, additional telomeric staining was detected on both arms of the medium-sized submetacentric chromosomes of the second pair (Figure 2b - inbox). The telomeric heterochromatic blocks were associated to the nucleolus organizer regions, in accordance with previous data for some other species of the genus (Koehler *et al.*, 1997; Molina *et al.*, 1998).

The heteromorphic sex chromosomes of both species followed the morphological and structural patterns of Z and W chromosomes previously reported in *Leporinus* species, with heterochromatic regions occupying nearly entirely the long arms of W chromosomes and the final portion of the long arms of Z chromosomes (Galetti Jr. *et al.*, 1981b; Koehler *et al.*, 1997; Molina *et al.*, 1998).

Interspecific differences related to constitutive heterochromatin evidenced by C-banding patterns have already been described in some *Leporinus* species. While some of them presented a low amount of heterochromatin, such as *L. piau*, others like *L. amblyrhyncus* and *L. taeniatus* present heterochromatic blocks distributed over centromeric and telomeric regions. Furthermore, in *L. striatus*, interstitial blocks of heterochromatin were also reported (Galetti Jr. *et al.*, 1991a). The different patterns of heterochromatin described in some species of this group suggest that some

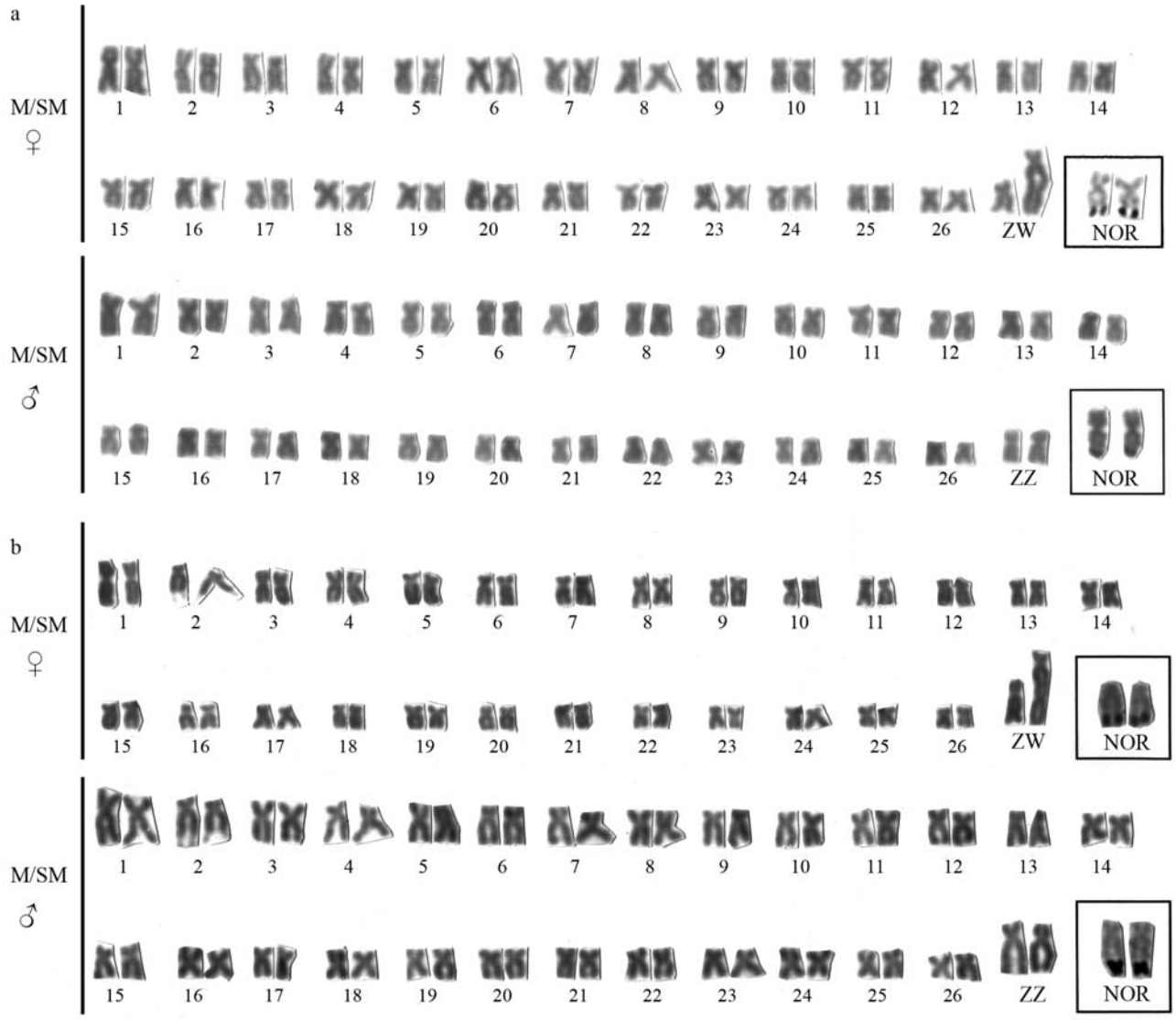


**Figure 1** - Giemsa-stained karyotype (female and male) of *Leporinus macrocephalus* (Piauçu) (a) and *Leporinus elongatus* (Piapara) (b). Inbox, the NOR-bearing chromosome pair 2.

rearrangements might have played a controlling role on the structural karyotypic changes (Galetti Jr. *et al.*, 1991a). Other species, with reduced amounts of heterochromatin, possibly presented a differentiation pattern associated with qualitative and quantitative changes in their heterochromatic segments (Galetti Jr. *et al.*, 1991b).

C-banding and NORs distribution allowed the characterization of chromosome markers in both parental species, which constitute important tools in the identification of the parental lineages and hybrids.

## Cytogenetic identification of the hybrid "Piaupara"

Twenty-one specimens of the hybrid "Piaupara" obtained through crosses between Piauçu females and Piapara males were analyzed. The results revealed a diploid number of 2n = 54 in all individuals, with a fundamental number of 108 and a chromosome formula composed of meta- and submetacentric chromosomes, besides ZZ/ZW sex chromosomes (Figure 3).

Due to the morphological similarity between the karyotypes of the parental species, the "Piaupara" hybrid presented the same diploid number and karyotypic formula described in *L. macrocephalus* (Piauçu) and *L. elongatus* (Piapara), thus preventing its identification. There are well documented cases of hybrids and their parental species sharing an identical karyotype, such as in hybrids between pacu (*Piaractus mesopotamicus*) and tambaqui (*Colossoma macropomum*), which have 2n = 54 and karyotypes composed of 20 metacentric and 34 submetacentric chromosomes (Almeida-Toledo *et al.*, 1987). A similar situation has been reported in natural hybrids resulting from crosses between *Cichla monoculus* and *Cichla temensis* by Brinn *et al.* (2004), who detected 2n = 48 acrocentric chromosomes in hybrids and parental specimens.

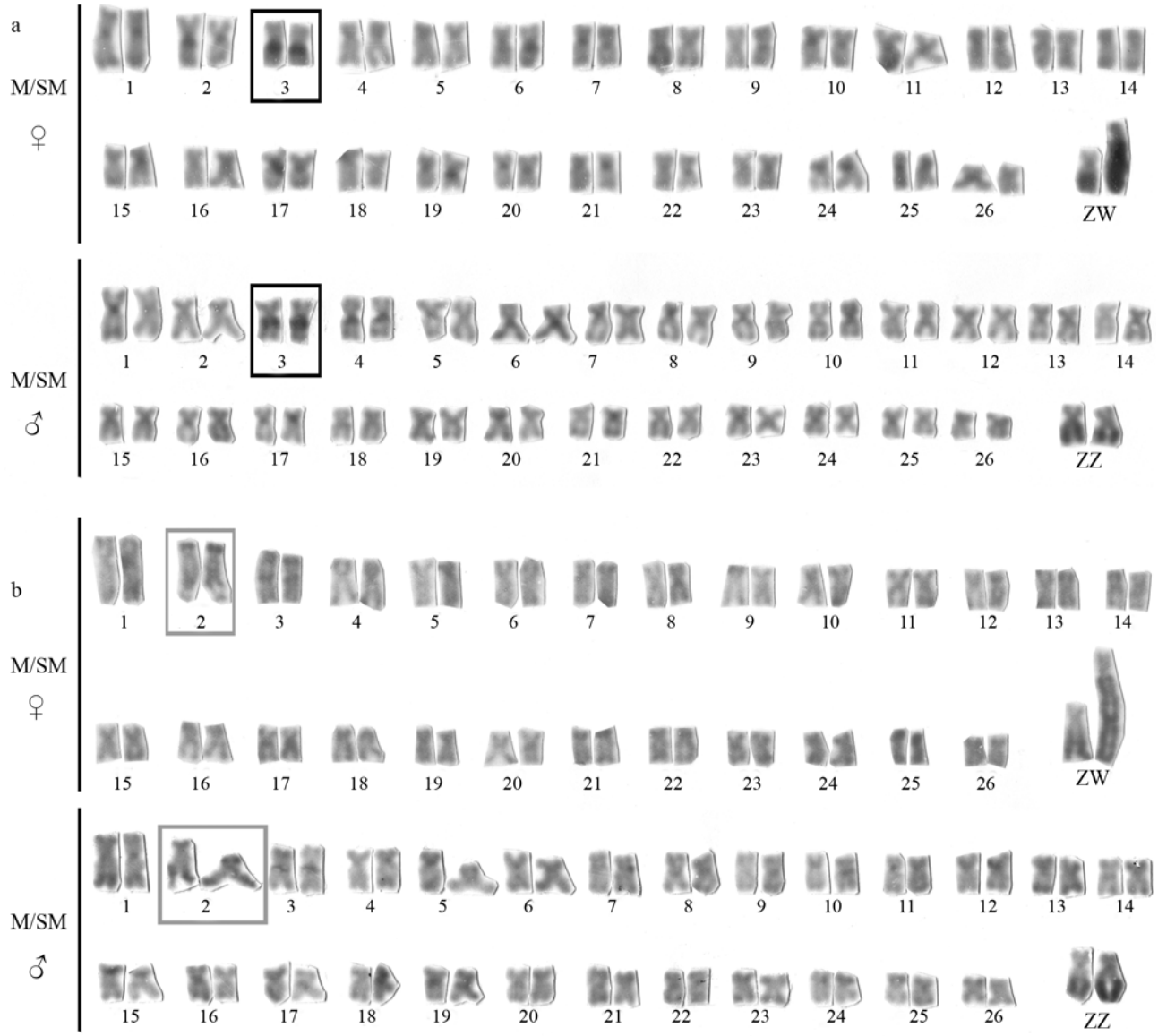


**Figure 2** - C-banded karyotypes (female and male) of *Leporinus macrocephalus* (Piauçu) (a) and *Leporinus elongatus* (Piapara) (b). The marker chromosomes are shown in detail (inbox).

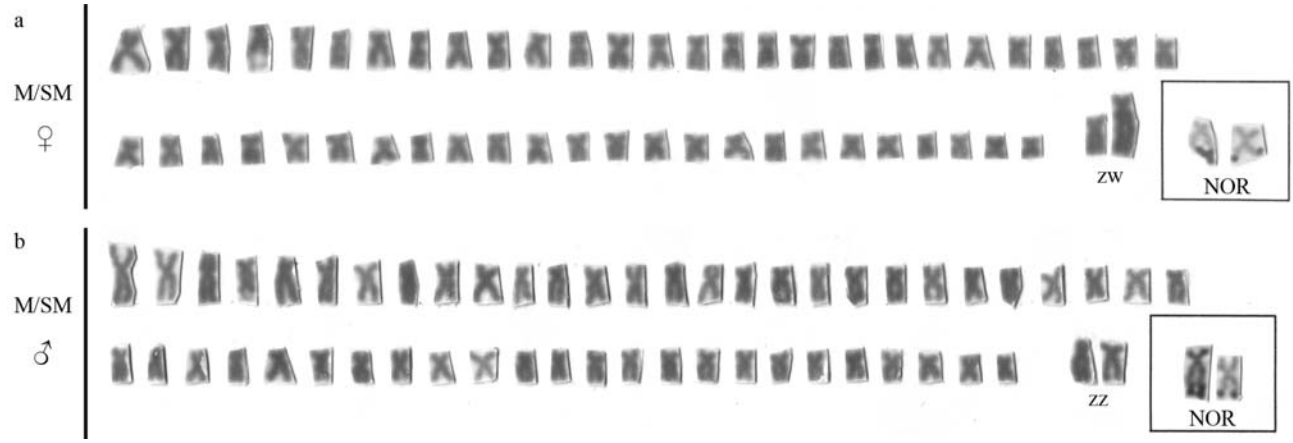


**Figure 3** - Karyotype of female (a) and male (b) individuals of the interspecific hybrid "Piaupara" (2n = 54). Inbox, the NOR-bearing chromosome pair.

In other cases of interspecific hybridization in fish, the parental species and their interspecific hybrids presented distinct karyotypes. That was the case of a hybridization program involving two species with 2n = 54 but distinct karyotypic formulas. Tambaqui (*Colossoma macropomum*) females were crossed with pacu-peva (*Mylossoma duriventris*) males and the crosses resulted in hybrid individuals with 54 chromosomes, which were identified by an acrocentric marker chromosome inherited from the male parental species (Kossowski *et al.*, 1983).

The cytogenetic analysis carried out in the hybrid "Piaupara" also revealed that the particular ZZ/ZW sex chromosome heteromorphism found in the parental species (Figure 3), is also present in the hybrid individuals and linked to sex determination.

In the hybrid individuals the submetacentric NOR-bearing chromosomes had different morphologies and were apparently not homologous (Figure 4). After silver nitrate staining the following NORs distribution was observed: 20,21% of the cells presented the NORs in the chromosomes of a submetacentric pair with different morphologies; 78,42% of the cells presented a NOR in one chromosome of the pair, identified as a component of the *L. elongatus* karyotype; and 1,37% of the cells presented the NOR in one chromosome identified as being from *L. macrocephalus* (Table 2 and Figure 5).

This variation in the NORs distribution is due to the fact that the silver nitrate staining detects active NORs, since it stains not the rDNA but rather a set of acidic proteins associated with the process of ribosome production (Howell, 1977; Jordan, 1987). Thus, the difference in activity found in the rDNA sites in the hybrids, which present a frequent activity of the NOR from the *L. elongatus* NOR-bearing chromosome, may indicate the occurrence nucleolar dominance. This phenomenon has already been identified in hybrids of cyprinid fishes (Gold *et al.*, 1991) and other organisms, such as in the wheat *Aegilops umbellulata* (Martini *et al.*, 1982), in *Xenopus* hybrids (Reeder and Roan, 1984), and in hybrids of *Drosophila* (Durica and Krider, 1978).

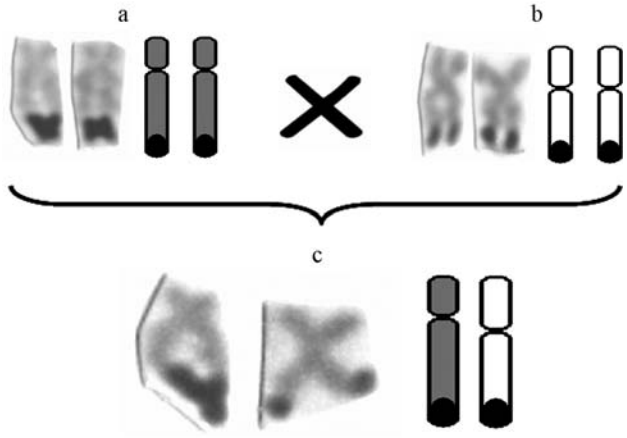


**Figure 4** - NOR-bearing chromosomes (silver nitrate staining) in the parental species *Leporinus elongatus* (a) and *L. macrocephalus* (b) and NOR-bearing chromosomes in the interspecific hybrid "Piaupara" (c).

C-banding in "Piaupara" revealed the presence of heterochromatic blocks at centromeric and pericentromeric regions of some chromosomes (Figure 6). Besides that, it showed an evident heterochromatic block near the peri-

**Table 2** - Distribution of NOR-bearing chromosomes after silver nitrate staining in the interspecific "Piaupara" hybrids.

| Cells with two Ag-NORs in the heteromorphic submetacentric pair (one chromosome from *L. elongatus* and the other from *L. macrocephalus)* | Cells with one Ag-NOR in the *L. elongatus* chromosome of the pair | Cells with one Ag-NOR in the *L. macrocephalus* chromosome of the pair | Total of analyzed cells (%) |
|---|---|---|---|
| 59 (20.21%) | 229 (78.42%) | 4 (1.37%) | 292 (100%) |

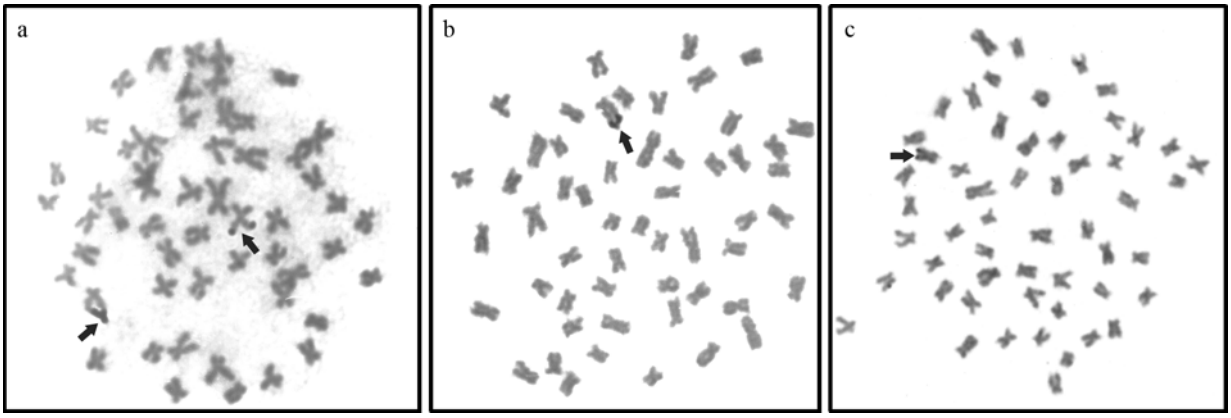


**Figure 5** - Metaphases of the interspecific hybrid "Piaupara" (silver nitrate staining). In (a), a heteromorphic submetacentric chromosome pair with one homologue from *L. elongatus* (arrow) and the other from *L. macrocephalus* (arrow); in (b), the *L. elongatus* homologue of the pair (arrows); and in (c), the *L. macrocephalus* homologue of the heteromorphic pair (arrows).

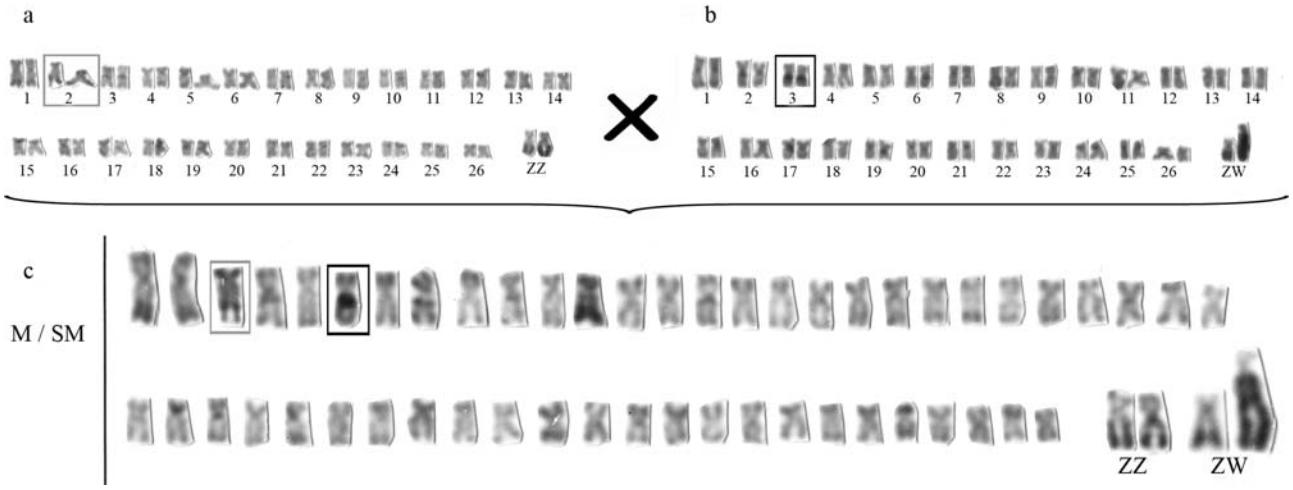


**Figure 6** - C-banded karyotypes of the parental species *Leporinus elongatus* (a) and *L. macrocephalus* (b) and of the interspecific hybrid "Piaupara" (c). The marker chromosomes from each parental species are shown in detail in the interspecific hybrid.

centromeric region of one chromosome (Figure 6 - inbox), analogous to that described in *L. macrocephalus*, and subtle staining at telomeric regions in both arms of one chromosome (Figure 6 - inbox), similar to that found in *L. elongatus*. Thus, the typical heterochromatic blocks from each parental species were present in the hybrid "Piaupara" in single chromosomes, demonstrating that chromosome features inherited from both parental species can be identified as specific parental markers in the hybrids.

C-banding also revealed that the hybrid sex chromosomes have the same morphological and structural patterns reported in both parental species, *L. macrocephalus* and *L. elongatus*, *i.e.*, almost entirely heterochromatic long arms in the W chromosome and heterochromatin in the final portion of the long arms of Z chromosomes (Figure 6).

When hybrids and parental individuals present similar karyotypes the use of differential staining techniques and chromosome banding are required to provide distinguishable chromosomal markers. C-banding allowed the precise identification of the parental species and the hybrids obtained from crosses between pacu (*Piaractus mesopotamicus*) and tambaqui (*Colossoma macropomum*) (Almeida-Toledo *et al.*, 1987, 1988). C-banding in the cichlid fish species *Cichla monoculus* and *C. temensis* and their hybrids revealed a very similar banding pattern distribution hindering the distinction between the parental and the hybrid specimens (Brinn *et al.*, 2004).

In the present work, Ag-NORs and C-banding proved to be informative and allowed the identification of specific chromosome markers of the parental sets in the hybrids. The use of chromomycin $A_3$ and *in situ* hybridization with the 18S probe will allow a better identification of the NORs sites in "Piaupara". It may also lead to a better comprehension of the process of nucleolar dominance that may be occurring in these individuals.

Cytogenetic markers can be very useful for the characterization and differentiation between parental species and artificial or natural interspecific hybrid lineages or individuals. Cytogenetic information can contribute to a better understanding of the dynamics of the interspecific hybridization process in fish, and provide support for hybridization projects and biological conservation programmes.

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