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Research Article

Cytogenetic analyses of two endemic fish species from the São Francisco River basin: *Conorhynchus conirostris* and *Lophiosilurus alexandri* (Siluriformes)

Marilza Barbosa de Almeida Marques[1], Orlando Moreira-Filho[2], Caroline Garcia[2] and Vladimir Pavan Margarido[3]

[1]*Fundação Municipal de Educação e Cultura de Santa Fé do Sul, Santa Fé do Sul, SP, Brazil.*
[2]*Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos, SP, Brazil.*
[3]*Centro de Ciências Biológicas e da Saúde, Universidade Estadual do Oeste do Paraná, Cascavel, PR, Brazil.*

Abstract

Two Siluriformes species endemic to the São Francisco River basin were characterized by conventional and differential cytogenetic analyses involving C-banding, Ag-nucleolar organizer region (NOR) and chromomycin A_3 (CMA_3) staining, and FISH (fluorescent *in situ* hybridization) with 18S and 5S rDNA probes. *Conorhynchus conirostris* presents a higher diploid number (2n = 60) than those detected in Pimelodidae representatives, whereas *Lophiosilurus alexandri*, with a karyotype of 2n = 54 chromosomes, presents a chromosomal constitution similar to that found in the family Pseudopimelodidae. Plesiomorphic characteristics such as single NORs at terminal positions are found in both species, as revealed by CMA_3 and silver nitrate staining, and FISH with a 18S rDNA probe. C-banding evidenced centromeric and telomeric heterochromatic blocks distributed over most of the chromosomes with a conspicuous heterochromatin segment in a pair of submetacentric chromosomes in *L. alexandri*. Such karyotype data, if compared to the cytogenetic pattern of other Siluriformes species, can be partially related to their degree of endemism, favorable to the occurrence and fixation of chromosomal rearrangements. The present study in representatives from these two Siluriformes families from the São Francisco River contributes to a better understanding of the karyotype evolution in species of this important order of Neotropical fishes.

Key words: incertae sedis, Pimelodidae, Pseudopimelodidae, rDNA, Siluriformes.

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Introduction

Endemism seems to be a common phenomenon along Brazilian hydrographic basins and appears widespread throughout several fish groups. Current taxa are endemic for two reasons: vicariance or dispersal. Generally, two major factors influence the degree of endemism of a given area, its isolation and climatic stability. Thus, a richness of endemic species can be found in areas that were isolated or stable over long periods of time (Cox and Moore, 2000).

Based on the distribution of Curimatidae fish species (Characiformes), Vari (1988) proposed a division of South American rivers at the Atlantic scope into eight main endemic areas, including the São Francisco River basin. The São Francisco River basin comprises the Brazilian States of Minas Gerais, Goiás, Bahia, Sergipe, Alagoas, Pernambuco, and the Federal District, corresponding to an area of 631,133 km^2 (Paiva, 1982). Its headwaters are located in the Canastra Hills, Southern Minas Gerais, and, after flowing through 2,700 km of the Brazilian territory, the river reaches the Atlantic Ocean between the States of Sergipe and Alagoas (PLANVASF, 1989).

The order Siluriformes constitutes a Teleostean group composed of 15 families and about 1,548 valid species in the Neotropical region (Reis *et al.*, 2003). According to Pinna (1998), Neotropical Siluriformes are composed by eight monophyletic groups: Diplomystidae, Cetopsidae, Loricaroidei, Doradoidea, and Aspredinidae, besides Pimelodinae, Pseudopimelodinae, and Rhamdiinae, formerly considered Pimelodidae subfamilies by Lundberg *et al.* (1991). However, considering the structural differences among the members of these families and the interrelationships with other Siluriformes groups, Pinna (1993, 1998) proposed a family status for the three latter groups:

Send correspondence to Orlando Moreira-Filho. Departamento de Genética e Evolução, Universidade Federal de São Carlos, Rodovia Washington Luiz km 235, 13565-905 São Carlos, SP, Brazil. E-mail: omfilho@power.ufscar.br.

Pimelodidae, Pseudopimelodidae, and Heptapteridae (= Rhamdiinae).

The family Pseudopimelodidae, comprising 26 species, is widespread over South America. As many species from this family are rare, a wider phylogenetic analysis is yet to be performed. The following genera are described for this family: *Batrochoglanis*, *Cephalosilurus*, *Lophiosilurus*, *Microglanis* and *Pseudopimelodus* (Shibatta, 2003).

The family Pimelodidae is one of the largest Siluriformes groups, comprising several species distributed throughout the Neotropical region. They present quite distinct morphology and size, and can be characterized by the presence of three pairs of barbels. Lundberg *et al.* (1991) recognized 32 genera for this family, including the genus *Conorhynchus*. In a recent review, Ferraris (2003) recognized only 31 genera in Pimelodidae, not including *Conorhynchus*, which is considered an *incertae sedis* in Siluriformes.

In 1981, Le Grande suggested that the ancestral karyotype of Siluriformes species was 2n = 56 (±2) and that the fundamental number (FN) was higher than 80. Oliveira and Gosztonyi (2000), studying *Diplomystes mesembrinus*, a representative of the most primitive family (Diplomystidae) of Siluriformes, revealed a diploid number of 56 chromosomes, suggesting this diploid chromosome number as the most basal in this fish order. Cytogenetic data indicate a wide chromosome variability among the species of this group, *i.e.*, the dispersal of chromosome numbers, if compared to the modal value, is quite frequent (Oliveira *et al.*, 1988b), which is probably related to speciation processes.

The diploid numbers found in the species ranged from 2n = 50 (*Calophysus macropterus*) (Ramirez-Gil *et al.*, 1998) to 2n = 58 chromosomes (*Pimelodus* cf. *maculatus*) (Augusto César Paes de Souza, personal communication) for Pimelodidae, and 2n = 54 chromosomes (*Microglanis cottoides*, *Pseudopimelodus bufonius*, *Pseudopimelodus mangurus*) (Vissoto *et al.*, 1999; Souza *et al.*, 2003; Martinez *et al.*, 2004) for Pseudopimelodidae, mostly meta- or submetacentric with high FN values, including some reports of supernumerary chromosomes in both families. The majority of the cytogenetic analyses carried out in these species is restricted to conventional staining techniques, particularly C and Ag-NOR bandings. The application of other methodologies, such as base-specific fluorochrome staining or fluorescent *in situ* hybridization (FISH) is extremely deficient in both groups.

In the present study, we carried out, for the first time, cytogenetic analyses in the species *Conorhynchus conirostris* and *Lophiosilurus alexandri*, both endemic to the São Francisco River basin.

Material and Methods

Sixteen specimens (11 males, three females, and two undetermined ones) of *C. conirostris*, and 56 specimens of *L. alexandri* (three males, four females, and 49 undetermined ones that presented undeveloped gonads) were provided by CODEVASF (Companhia de Desenvolvimento do Vale do Rio São Francisco) from the Três Marias region (São Francisco River basin, Brazil) and used for cytogenetic analyses.

The mitotic chromosomes were obtained by air-drying (Bertollo *et al.*, 1978; Foresti *et al.*, 1993). The silver stained nucleolar organizer regions (Ag-NORs) were obtained according to the procedure described by Howell and Black (1980). Heterochromatin was detected by C-banding according to Sumner (1972), with slight modifications. Chromomycin A_3 (CMA_3) staining was performed according to Schweizer (1980).

Fluorescent *in situ* hybridization (FISH) using 18S and 5S rDNA probes was carried out according to Pinkel *et al.* (1986), with modifications. The 18S rDNA probe was obtained from the fish *Prochilodus argenteus* (Hatanaka and Galetti, 2004), and the 5S rDNA probe from *Leporinus elongatus* (Martins and Galetti, 1999). The probes were labeled with biotinylated adenine (16-dATP) by nick translation, according to the manufacturer's instructions (Bionick Labeling System, Invitrogen).

Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a), according to arm ratio (Levan *et al.*, 1964). The fundamental number (FN) was calculated considering m, sm, and st chromosomes as biarmed elements and a as one-armed chromosomes.

Results

In *C. conirostris*, a diploid number of 2n = 60 was detected. The karyotype is composed of 20m + 18sm + 10st + 12a (FN = 108), without any difference between sexes (Figure 1a). The single Ag-NORs were terminally located on the long arms of a subtelocentric chromosome pair (Figure 2a), and constitutive heterochromatin was detected on the centromeric and telomeric regions of most of the chromosomes (Figure 2c).

Analysis with a GC-specific fluorochrome revealed CMA_3^+ sites at the nucleolar organizer regions, equivalent to Ag-NOR marks, lacking any additional site (Figure 3a). FISH results with the 18S rDNA probe were coincident to those obtained by silver and CMA_3 staining, thus confirming the occurrence of a single NOR system. There was no size difference between the NOR-bearing homologues (Figure 3b). The 5S rRNA genes in this species were detected by FISH, revealing positive signals on the short arms of a single pair of acrocentric chromosomes (Figure 3c).

A diploid number equal to 54 chromosomes was established for *L. alexandri*. The karyotype formula was 16m + 18sm + 10st + 10a (FN = 98), without any evident sex-related chromosome differentiation (Figure 1b). Single Ag-NORs were present on the short arms of a single sm chromosome pair, with frequent association between the

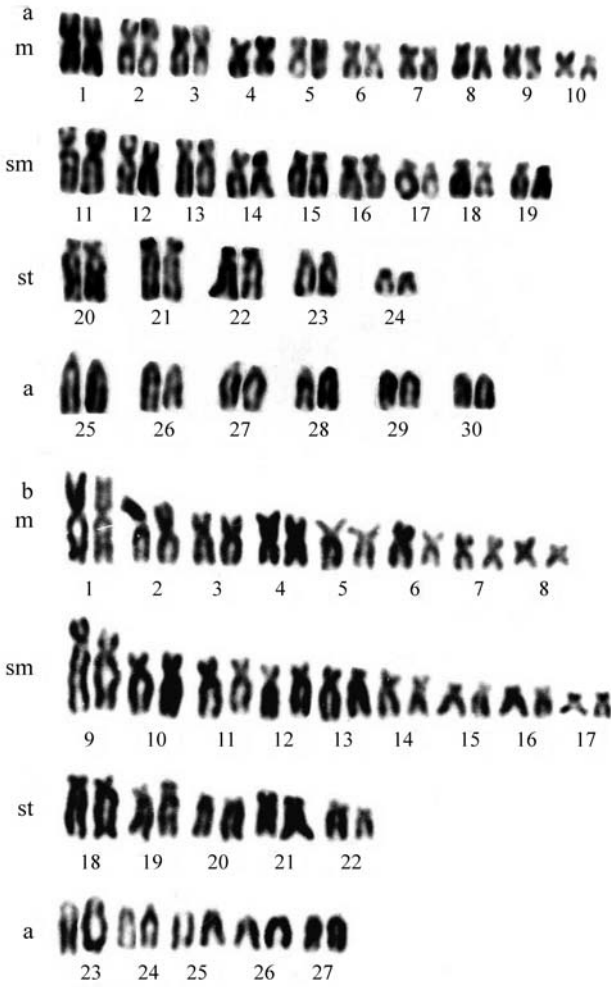

Figure 1 - Conventional Giemsa stained karyotypes of (a) *Conorhynchus conirostris*, and (b) *Lophiosilurus alexandri*.

homologues (Figure 2c), and constitutive heterochromatin was detected on most chromosomes in the centromeric region, besides an entirely heterochromatic sm pair with long arms (Figure 2d).

The CMA_3 staining evidenced positive signals on the short arms of a sm pair similar to the silver staining results (Figure 3d). The 18S rRNA genes were located on the short arms of sm chromosomes, equivalent to the Ag-NOR and CMA_3 markings. Moreover, size differences between the NOR-bearing homologous chromosomes were detected, characterizing a structural heteromorphism (Figure 3e). FISH with the 5S rDNA probe revealed these cistrons on the long arms of a sm chromosome pair (Figure 3f).

Discussion

Siluriformes represents the second largest fish order (Characiformes is the first) in the South American hydrographic basins (Fink and Fink, 1981; Oliveira and Gosztonyi, 2000). Several species have remarkable economic relevance and aquaculture potential (Sato and Godinho, 1988). Therefore, they have been extensively studied over the last decades, especially regarding genetic and systematic approaches and, despite controversy over phylogenetic relationships between species, genera, and families in this order, there has been an increase in available information (Nelson, 1994; Pinna, 1993, 1998).

Srudies by Pinna (1993, 1998) suggested a family status for Pseudopimelodidae, apart from the family Pimelodidae. Taken together, the cytogenetic data obtained up till now for Pimelodidae and Pseudopimelodidae present a chromosome pattern compatible to that proposed for the order, with some exceptions in more divergent species (Oliveira *et al.*, 1988a). On the other hand, the karyotype differences found among and within some species reinforce the hypothesis that both families do not constitute a natural fish group (Pinna, 1998). Furthermore, some species whose taxonomical units are still to be properly identified as complexes are also indicated, just as suggested for some other fish groups comprising small and isolated populations, such as the characin *Astyanax scabripinnis* (Moreira-Filho and Bertollo, 1991).

The Pseudopimelodidae species analyzed thus far exhibit karyotypes with 2n = 54 chromosomes (Vissoto *et al.*, 1999; Souza *et al.*, 2003; Martinez *et al.*, 2004). *Lophiosilurus alexandri* analyzed in the present study also exhibits a diploid number of 54 chromosomes. Such homogeneity on karyotype structure, slightly different from that found in most Pimelodidae representatives, corroborates the current systematic classification (Pinna, 1998). Most of the differences observed among the karyotypes of species belonging to this family are related to chromosome structure, with no alteration in the diploid number. Such variations can be caused by non-Robertsonian rearrangements (inversions), heterochromatin addition, or discrepancies among chromosome measurements performed by distinct authors.

The other species analyzed in the present work, *Conorhynchus conirostris*, although recognized as Pimelodidae by Lundberg *et al.* (1991), was recently considered as an *incertae sedis* in Siluriformes (Ferraris, 2003). *Conorhynchus conirostris* exhibits a diploid number of 60 chromosomes, a higher diploid value than those detected in the family Pimelodidae. The deviation in karyotype seen in *C. conirostris* could has been favored by the endemism of this species (Fowler, 1951; Travassos, 1960), comprising isolated populations exposed to peculiar selective pressures in their environment. If we assume that endemic populations are subject to a higher degree of inbreeding than the widely distributed (panmitic) ones, such effects could be maximized. The origin of such a karyotype constitution apparently involved Robertsonian rearrangements such as centric fission, leading to the numerical (and structural) chromosome variations observed in this species. Therefore, the fixation of a new chromosome rearrangement in this species would be easily accomplished, increas-

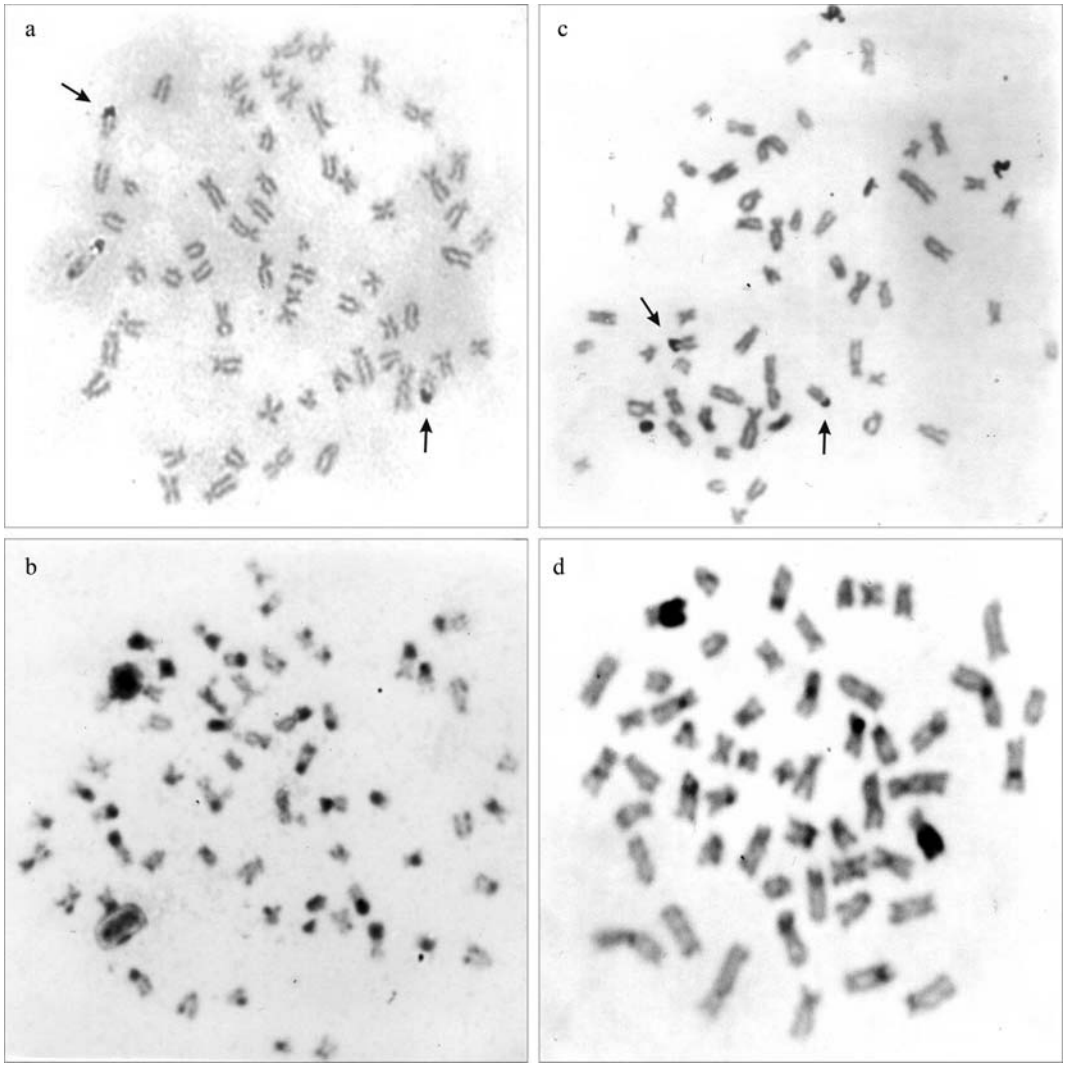

Figure 2 - Metaphases of *Conorhynchus conirostris*: (a) silver nitrate staining, and (b) C-banding; and metaphases of *Lophiosilurus alexandri*: (c) silver nitrate staining, and (d) C-banding. The Ag-NORs are indicated by arrows (a, c).

ing the diploid number and differing from the common pattern observed for Pimelodidae, and corroborating the hypothesis of Ferraris (2003) that placed *C. conirostris* as an *incertae sedis* in Siluriformes.

Usually, the C-banding pattern observed in species of the families Pimelodidae and Pseudopimelodidae is discrete, involving small chromosome portions in centromeric, pericentromeric, and/or telomeric positions. *C. conirostris* followed this tendency, as it presented positive C-bands restricted to centromeric and telomeric regions of most chromosomes. On the other hand, *L. alexandri* presented a pair of entirely heterochromatic submetacentric chromosomes besides the heterochromatin segments distributed over centromeric and telomeric regions,. The detection of this apomorphy can be correlated to ecological peculiarities of this species, which is also endemic to the São Francisco River basin (Fowler, 1951; Travassos, 1959). Reports on heterochromatin variation in fish species (Mantovani *et al.*, 2004) or other vertebrate groups (Pathak *et al.*, 1973) are usually associated to restricted and small populations, susceptible to higher rates in chromosome evolution (King, 1987).

Conorhynchus conirostris presented NORs located at a terminal position on the long arms of a subtelocentric pair, whereas *L. alexandri* showed positive terminal marks on the short arms of a submetacentric pair. It should be pointed out that a size difference between the NOR-bearing homologues was frequently observed in *L. alexandri* either by FISH or by silver nitrate staining, characterizing a structural heteromorphism. Such a difference in copy number of ribosomal genes between homologous chromosomes can be caused by several mechanisms common in repetitive DNA regions, such as unequal exchange at meiosis or in tandem amplifications (Smith, 1976; Insua *et al.*, 1999), and they constitute an additional source of microstructural

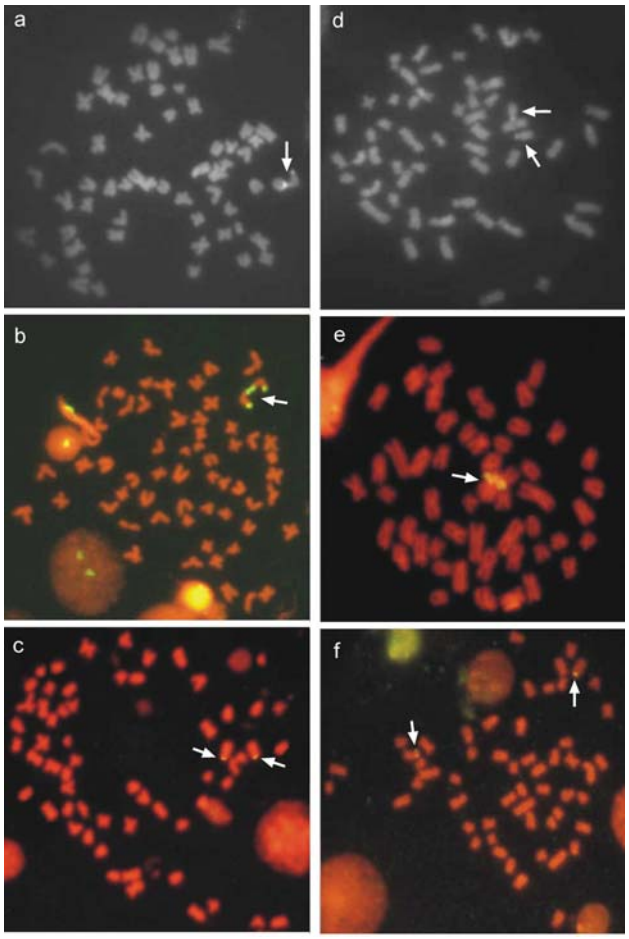

Figure 3 - Metaphases of *Conorhynchus conirostris*: (a) chromomycin A_3 staining, (b) 18S rDNA-FISH, and (c) 5S rDNA-FISH; and metaphases of *Lophiosilurus alexandri*: (d) chromomycin A_3 staining, (e) 18S rDNA-FISH, and (f) 5S rDNA-FISH. The chromosomes evidencing positive bands by chromomycin A_3 staining, and by 18S and 5S rDNA-FISH are indicated by arrows.

variability, especially frequent in species with single NORs (see Feldberg *et al.*, 1999; Wasko and Galetti, 2000).

In the same way, the presence of GC-rich heterochromatic blocks equivalent to NOR sites, as evidenced by CMA_3 staining, is practically a constant characteristic of fish species (Phillips and Hartley, 1988; Schmid and Guttenbach, 1988; Sola *et al.*, 1992), with very few exceptions (Souza *et al.*, 1996; Margarido and Galetti, 2000; Mantovani *et al.*, 2004).

The 5S rRNA genes are localized on a single chromosome pair in both species, and both classes of rDNA (45S and 5S) are not syntenic. Several studies indicate that this rDNA organization could be a trend in Neotropical fishes (Long and David, 1980; Vicente *et al.*, 2001, among others). It is believed that such a distribution is a primitive condition of the fish genome (Martinez *et al.*, 1996). The presence of two 5S rDNA-bearing pairs displaying independent evolutionary pathways seems to be a rule in most fish species (Martins and Galetti, 2001). However, just like in the present work, exceptionally species that present a single pair with 5S rRNA genes are found. While the number of reports on 5S rDNA localization in fish species is still low in the literature, especially amongst Siluriformes, making further considerations rather difficult.

The data obtained in the present study reinforce the idea that endemism plays an important role in the speciation process, capable of determining genetically differentiated populations under the effects of local selection. This would then lead to the origin of distinct biological units (Cox and Moore, 2000), possibly reflected by morphological and/or cytological features.

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