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Basic and molecular cytogenetics in freshwater Cichlidae (Osteichthyes, Perciformes). Karyotypic conservationism and divergence

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Abstract — The chromosomes of one *Cichlasoma facetum* and three *Geophagus brasiliensis* populations from the headwaters of adjacent river basins (Paraná State, southern Brazil), were investigated using differential staining techniques (C-banding, Ag-NORs, DAPI and CMA_3) and fluorescent *in situ* hybridization (FISH) with 18S rDNA and 5S rDNA probes. The diploid chromosome number (2n) was invariably 48 in the four populations, with karyotypes composed of 3 submetacentric and 21 subtelo/acrocentric chromosome pairs in *G. brasiliensis* and 5 submetacentric and 19 subtelo/acrocentric chromosome pairs in *C. facetum*, with no heteromorphic sex chromosomes. The differences detected in the FN numbers between *G. brasiliensis* (FN=54) and *C. facetum* (FN=58) indicate that pericentric inversion is the probable rearrangement that led to the cytogenetic divergence between these species. The overall karyotype similarity strongly suggests a close kinship among the three *G. brasiliensis* populations, despite a distinct C-banding pattern showed by the Jaguariaíva river population. The data suggest that dispersion events were responsible for the present fish distribution in the river basins analyzed. The divergence in the C-banding pattern is probably due to gene flow restriction between populations during their geological/evolutionary history.

Key words: biogeography; Cichlidae fish; heterochromatin; karyotypic evolution; NORs

INTRODUCTION

The order Perciformes has the highest number of species among the Teleostei, 14% of them inhabiting freshwaters. According to Brum (1995), karyotypic analyses of 420 representatives from 50 Perciformes families evidence that 67% (283 species) have a diploid number (2n) of 48 chromosomes, 30% (124 species) have 2n<48 chromosomes and 3% (13 species) have 2n>48 chromosomes. The characters 2n=48 acrocentric chromosomes and consequent fundamental number (number of chromosome arms) of 48 seem to be the basal characteristics for the order Perciformes (Brum and Galetti Jr. 1997), especially concerning the marine groups.

In spite of the karyotype with 48 acrocentric chromosomes being the most frequent among cichlids, the diploid number may vary between 2n=32 and 2n=60 chromosomes (Feldberg *et al.* 2003). The bimodal distribution of the diploid numbers becomes evident when related with the geographic distribution of the species. African cichlids have a modal diploid number of 44 chromosomes, with a variation from 32 to 48 chromosomes and a FN between 44 and 88. In turn, neotropical cichlids present a modal 2n=48 chromosomes, with a variation from 38 to 60 chromosomes and a FN varying from 44 to 118 (Feldberg *et al. op. cit.*).

Geophagus brasiliensis and *Cichlasoma facetum* are two of the most common freshwater cichlid species of southern and southeastern Brazil. They are found in a great variety of Brazilian aquatic biomes and, in a few cases, morphological differences between the populations are pronounced to the point of suggesting the existence of different species. Thus, the karyotypes of one *C. facetum* and three *G. brasiliensis* populations from the headwaters region of three important rivers in Paraná State (Tibagi, Ribeira and Paranapanema

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rivers), which are closely located but separated by geographical barriers, were analyzed in the present study, seeking to establish possible evolutionary/biogeographical relationships between the populations and species involved.

MATERIAL AND METHODS

Sixty *Geophagus brasiliensis* specimens were analyzed: 15 (8 males and 7 females) from the Jaguariaíva river – subaffluent of the Paranapanema river (24° 35‘ 42” S and 49° 25‘ 67” W), 22 (12 males and 10 females) from the Socavão river-subaffluent of the Ribeira river (24° 53‘ 31” S and 49° 35‘ 37” W) and 23 (10 males and 13 females) from the Verde river – subaffluent of the Tibagi river (25°04’81” S and 50°04’63” W). Sixteen *Cichlasoma facetum* specimens (8 males and 8 females) from the Tarumã Lake (Upper Tibagi river basin), Vila Velha State Park, Ponta Grossa, Brazil (25° 14’ 09” S and 50° 00’ 17” W) were also analyzed (Figure 1). Testimony samples were deposited in the Museu de Zoologia (Zoology Museum) of the Universidade Estadual de Londrina (Londrina State University), Paraná, Brazil (voucher number 1735).

The chromosome preparations were obtained from anterior kidney cells using the *in vivo* colchicine treatment (Bertollo *et al.* 1978). Heterochromatin was detected by the C-banding method (Sumner 1972), while the nucleolar organizing regions (NORs) were detected using silver nitrate staining - Ag-NORs (Howell and Black 1980), as well as by double fluorochrome staining using chromomycin A_3+DAPI (Schweizer 1980), which are indicative of GC- and AT-rich regions, respectively.

Fluorescent *in situ* hybridization (FISH) was used to map the rDNA genes in the chromosomes. Two probes were utilized, one for 18S rDNA (approximately 1.800 bp), obtained by PCR from the nuclear DNA of *Prochilodus argenteus* (Hatanaka and Galetti Jr. 2004), and another for 5S rDNA, obtained from *Leporinus elongatus* (Martins and Galetti Jr. 1999). The probes were marked with 14-dATP biotin by nick translation, following manufaturer’s instructions (Bionick Labelling System – Invitrogen). The metaphase chromosomes were treated according to the procedure described by Pinkel *et al.* (1986) and analyzed in an Olympus BX50 epifluorescence microscope. The chromosome figures were captured using the Image Pro-Plus software (Media Cybernetics).

The chromosomes were organized into three groups in the karyotype, i.e., submetacentric (sm), subtelocentric (st) and acrocentric (a), depending on their arm ratios (Levan *et al.*, 1964), and arranged in a decreasing order of size. The number of chromosome arms (fundamental number - FN) was calculated taking into account the st/a and the sm chromosomes with one and two arms, respectively.

RESULTS

Karyotypic structure - The samples from the three *G. brasiliensis* populations (Jaguariaíva, Socavão and Verde rivers) and the single *C. facetum* population (Tarumã Lake) presented a karyotype constituted of 2n=48 in both males and females, making them homomorphic, that is, with no morphologically differentiated sex chromosomes. In *G. brasiliensis*, the fundamental number was 54, with the occurrence of 3 submetacentric and 21 subtelo/acrocentric chromosome pairs being verified. In *C. facetum*, FN=58 was observed due to the occurrence of 5 submetacentric and 19 subtelo/acrocentric chromosome pairs (Figure 1).

C-positive bands located preferentially in centromeric/pericentromeric regions were evidenced in *G. brasiliensis* as well as in *C. facetum* (Figure 1c, d, g, h). However, the *G. brasiliensis* population from the Jaguariaíva river presented a larger quantity of heterochromatin when compared to the Socavão and Verde river populations, besides having many chromosomes with interstitial heterochromatic segments (Figure 1 c, d, g).

Ag-NOR, CMA_3 and DAPI bandings and localization of the 18S and 5S ribosomal genes - The nucleolar organizing regions, evidenced by silver nitrate (Ag-NORs) and fluorescent *in situ* hybridization with an 18S rDNA probe, were located in the telomeric region of the short arm of st/a pair 6 for the three *G. brasiliensis* samples (Figures 1a, b, e, and 3a, b, e) and in the telomeric region of the short arm of st/a pair 8 for *C. facetum* (Figures 1f and 3f). A size heteromorphism of the 18S site was occasionally observed in the *G. brasiliensis* populations, always in heterozygosis (Figure 4). The double CMA_3/DAPI staining showed a single CMA_3 positive and DAPI negative band in accordance to the NOR site (Figures 2 and 4). The 5S rDNA site was located in the interstitial region of the long arm of st/a pair 10 for the three *G. brasiliensis* samples (Figure 3c, d, g) and in the inter-

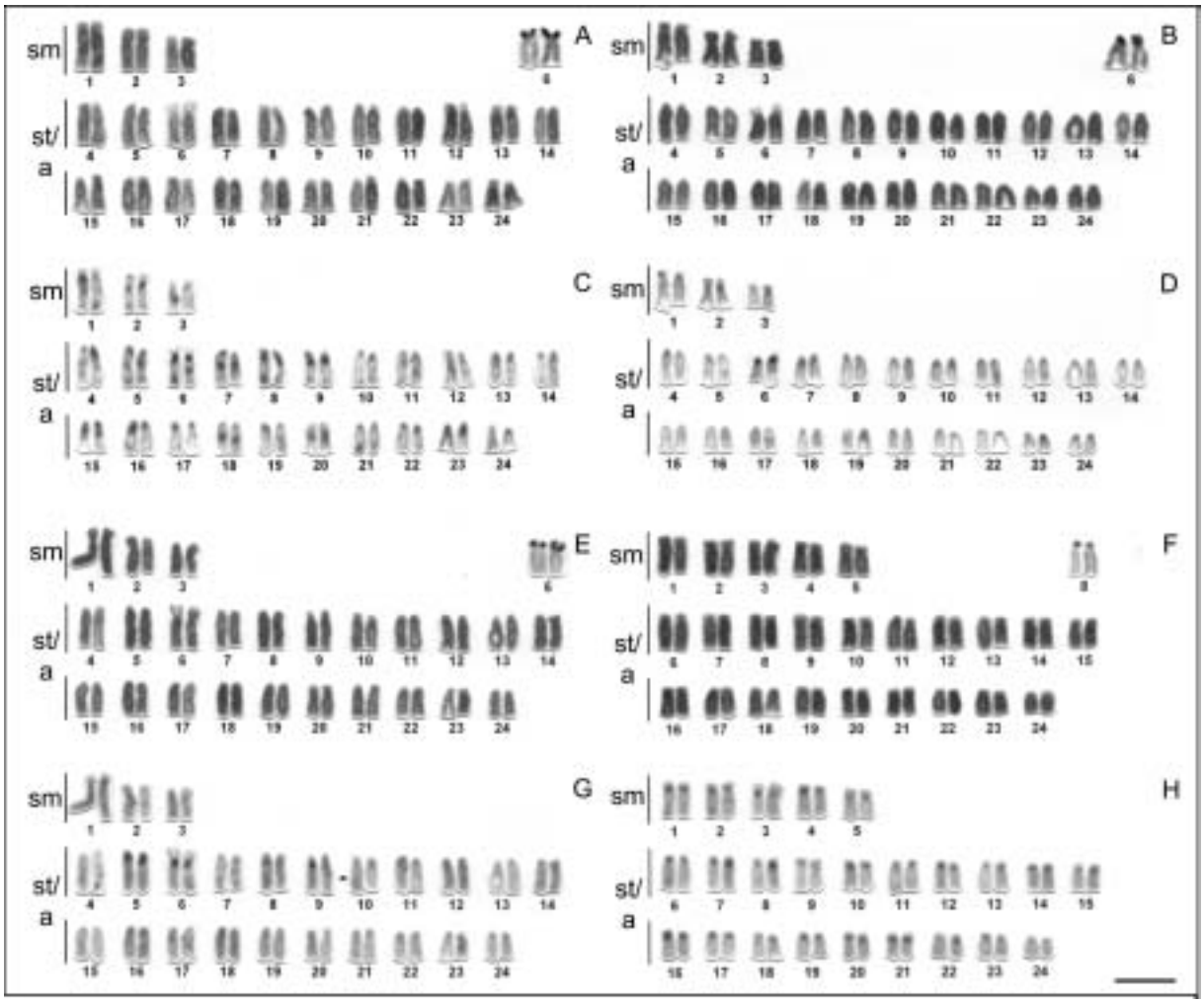

Fig. 1 — *Geophagus brasiliensis* (A, B, E) and *Cichlasoma facetum* (F) karyotypes with conventional Giemsa staining, and sequential heterochromatin analysis (C, D, G) and (H), respectively. The chromosome pair bearing the Ag-NOR-positive site is highlighted. (A, C) Jaguariaíva river population; (B, D) Socavão river population; (E, G) Verde river population. Bar represents 5 μm.

stitial region of the long arm of st/a pair 11 for *C. facetum* (Figure 3h).

DISCUSSION

The family Cichlidae possesses approximately 99 nominal species with described karyotypes, of which 68 present a diploid number equal to 48 chromosomes, 27 present less than 48 chromosomes and only 4 present more than 48 chromosomes (Brum and Galetti Jr. 1997), the two extreme values being represented by 2n=32 and 2n=60 chromosomes, respectively (Feldberg *et al.* 2003). In truth, 2n=48 acrocentric chromosomes and, consequently, FN=48 seem to be the basal characteristics for the order Perciformes as a whole (Galetti Jr. *et al.* 2000), especially concerning marine groups. Thus, the presence of 2n=48 in the *G. brasiliensis* and *C. facetum* populations here studied represents the maintenance of a plesiomorphic condition in these groups, corroborating a few analyses previously performed (Feldberg and Bertollo 1985a; Brum *et al.* 1998; Feldberg *et al.* 2003). On the other hand, pericentric inversions are considered the main rearrangements implicated in the diversification of the ancestral 2n=48 of the Perciformes (Galetti Jr. *et al.* 2000; Affonso and Galetti Jr. 2005), as evidenced by Feldberg and Bertollo (1985a), Oliveira *et al.* (1988), Salas and Boza (1991), Martins *et al.* (1995), Brum *et al.* (1998) and Affonso and Galetti Jr. (2005). This same event may also elucidate the different fundamental numbers found in the present analysis, where the

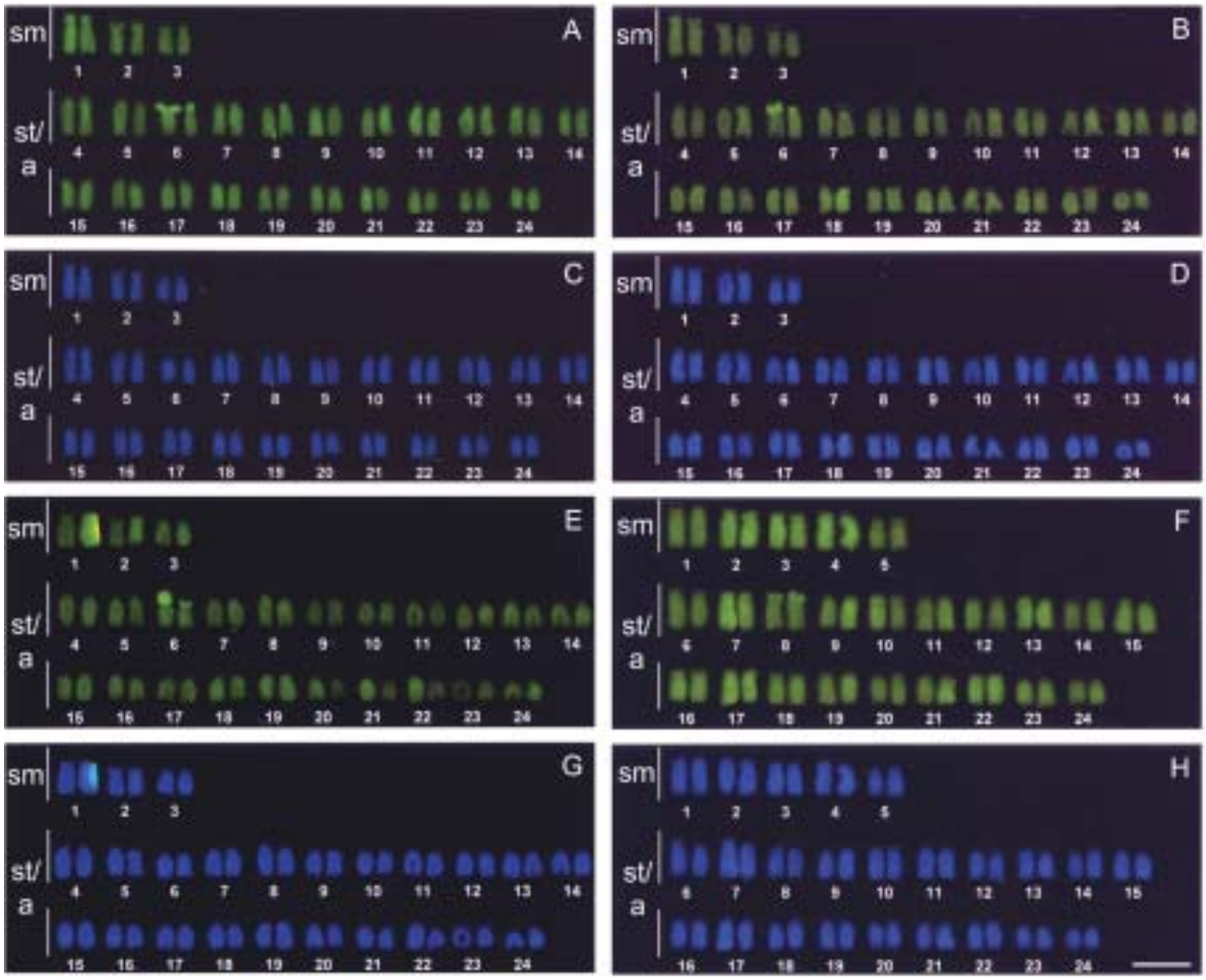

Fig. 2 — *Geophagus brasiliensis* (A, B, E) and *Cichlasoma facetum* (F) karyotypes stained with the chromomycin A_3 fluorochrome and sequentially stained with the DAPI fluorochrome (C, D, H) and (H), respectively, showing a GC-positive and AT-negative site in the 6th chromosome pair of *G. brasiliensis* and 8th of *C. facetum*. (A, C) Jaguariaíva river population; (B, D) Socavão river population; (E, G) Verde river population. Bar represents 5 μm.

G. brasiliensis populations possess FN=54 (6 sm + 42 st/a) and *C. facetum* FN=58 (10 sm + 38 st/a). In fact, the variation in the number of arms has been a good chromosomal marker for many cichlid species.

There are few comparative studies between Cichlidae populations concerning heterochromatin, probably due to the fact that heterochromatic blocks are practically restricted to the pericentromeric region of the chromosomes (Kornfield *et al*. 1979; Oliveira and Wright 1998). However, despite the maintenance of this same general pattern of heterochromatin distribution in the species/populations here studied, *G. brasiliensis* of the Jaguariaíva river showed a peculiar C-banding pattern, since it presents many chromosome pairs with conspicuous and interstitial heterochromatic blocks not found in the other populations of this species, as well as in *C. facetum*. Furthermore, the quantity of heterochromatin present in the chromosomes of *G. brasiliensis* of the Jaguariaíva river is much larger than the total observed in the populations of the Socavão and Verde rivers, which, in turn, show a very mutually similar pattern. Coincidently, the populations of the Socavão and Verde rivers are more closely situated than the Jaguariaíva river population, which, considering a probably larger geographic isolation between this latter population and the others, could justify the observed differences.

The chromosome pair bearing the nucleolar organizing regions (Ag-NORs, 18S rDNA sites) is coincident in the three *G. brasiliensis* populations (pair 6, st/a), very likely being homeologous to the NOR-bearing pair of *C. facetum* (pair 8, st/a). These same chromosome pairs evidence a

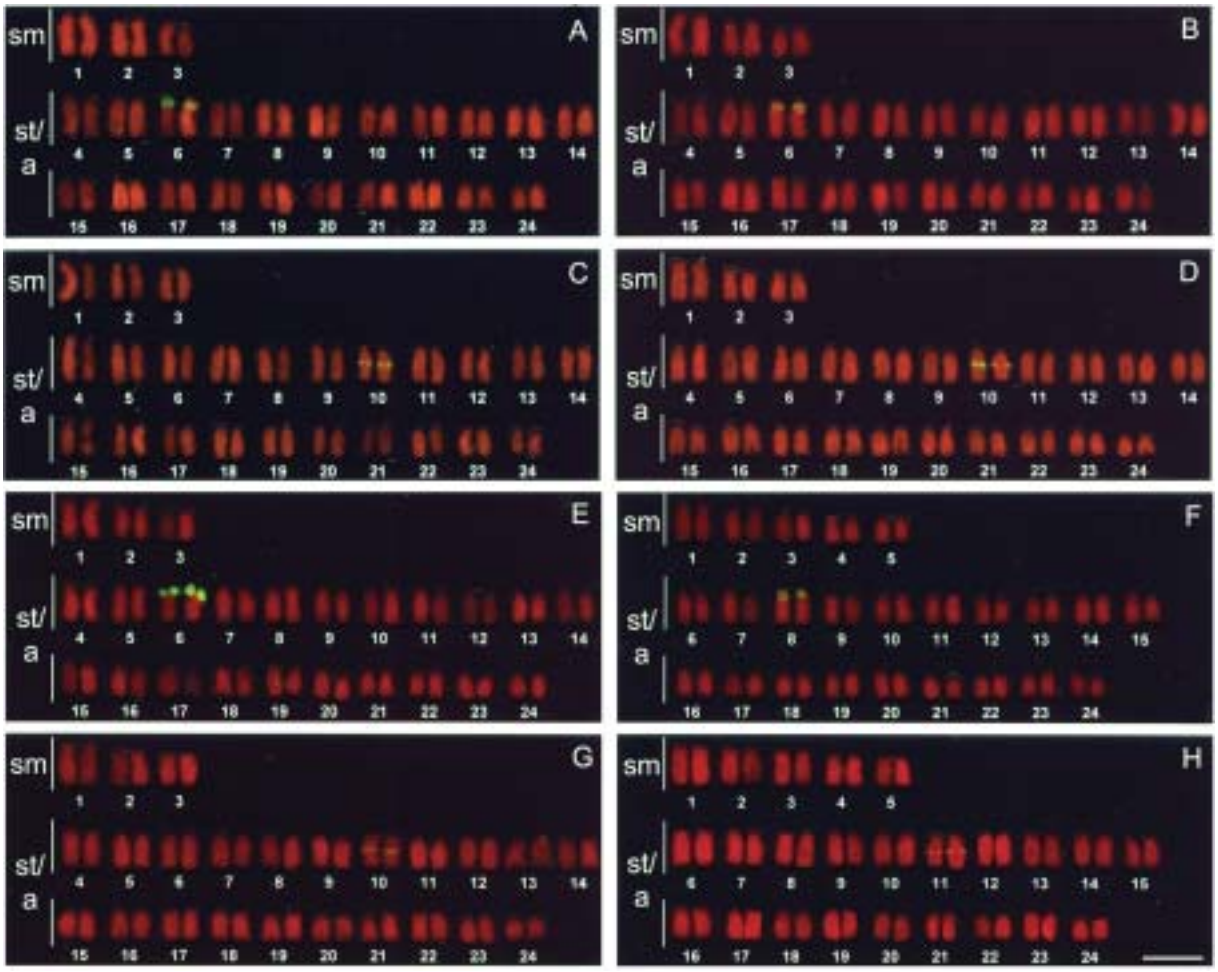

Fig. 3 — *Geophagus brasiliensis* (C, D, G) and *Cichlasoma facetum* (F) karyotypes showing the localization of the 18S rDNA in chromosome pairs 6 and 8, respectively, and the localization of the 5S rDNA in chromosome pairs 10 (C, D, G) and 11 (H), respectively. (A, C) Jaguariaíva river population; (B, D) Socavão river population; (E, G) Verde river population. Bar represents 5 μm.

CMA_3-positive region coincident with the NOR site, reinforcing its constitution rich in GC base pairs and, alternatively, poor in AT bases. Among fish (Pendás *et al.* 1993; Galetti Jr. 1998; Almeida-Toledo 1998; Vicari *et al.* 2003; 2005) and amphibians (Schmid 1982), NORs generally present themselves as positive sites for the GC-specific fluorochromes such as chromomycin and mitramycin. Nevertheless, it is also known that regions stained with chromomycin/mitramycin may not correspond to rDNA loci, but only to GC-rich heterochromatin (Martinez *et al.* 1991; Artoni and Bertollo 1999). In the present case, in both *Geophagus* and *Cichlasoma*, a heterochromatic block situated directly underneath the NOR site in chromosomes 6 and 8, respectively (Figures 1, 4) is identified, showing that the ribosomal genes are not interspersed with heterochromatin but located adjacent to it, in a fashion similar to a few other fish species (Artoni and Bertollo 1999).

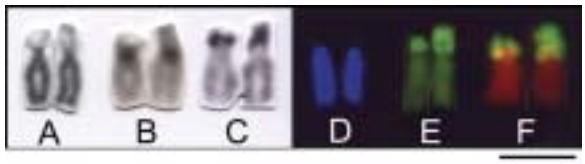

Fig. 4 — Chromosome pair number 6 of *Geophagus brasiliensis*, showing the NOR localization in the short arm and its size polymorphism under different analysis methodologies: (A) conventional Giemsa staining, evidencing a very conspicuous secondary constriction; (B) C-banding, showing the localization of the heterochromatin adjacent to the NOR site; (C) Ag-NOR-positive site; (D) DAPI-negative site; (E) chromomycin A_3-positive site; and (F) 18S rDNA localization. Bar represents 5 μm.

The family Cichlidae, with rare exceptions, is characterized for presenting simple NORs (a sin-

gle pair) located in relatively large chromosomes (Feldberg *et al.* 2003), corresponding to a probable symplesiomorphism for this group (Feldberg and Bertollo 1985b). Hsu *et al.* (1975) already considered simple NORs, in comparison with multiple NORs, as a more primitive state of rDNA distribution on the karyotypes. On the other hand, together with the occurrence of simple NORs, the occurrence of size heteromorphism between the homologous NOR sites is also frequent (Feldberg and Bertollo 1985b; Brum *et al.* 1998; present work), which may be due to uneven permutations during meiosis and/or exchanges between sister chromatids involving repeated sequences of these regions. This heteromorphism was clearly observed in the three *G. brasiliensis* populations here studied, where one of the NOR sites was duplicated in relation to its homologue.

The 5S rDNA-bearing chromosomes in *G. brasiliensis* and *C. facetum*, st/a pairs 10 and 11, respectively, are probably homeologous as well, showing interstitial sites in the long arm, and therefore not syntenic to the 18S rDNA sites. Among the Perciformes, there are still few studies on the localization of the 5S rDNA. Inafuku *et al.* (2000) located these genes in the short arm of two chromosome pairs in *Acheilognathus tabira* and in four pairs of *Cyprinus carpio*, also verifying that the NOR and 5S rDNA site are syntenic in *A. tabira*. An unusual situation where the ribosomal 5S genes are superposed to a chromomycin A3-positive region was detected in some Perciformes species (Deiana *et al.* 2000; Affonso and Galetti Jr. 2005). In *Centropyge aurantonotus*, eighteen 5S rDNA sites were detected, many of them possibly corresponding to inactive pseudogenes (Affonso and Galetti Jr. *op. cit*).

G. brasiliensis and *C. facetum* maintain many characteristics considered plesiomorphic for Perciformes, such as a diploid number of 48, a predominance of st/a chromosomes in the karyotype and number and localization of NORs. The high karyotypic conservationism between the three *G. brasiliensis* populations studied in relation to karyotypic macrostructure as well as to some banding patterns reinforces the proposition that dispersion events, probably from headwater captures, may have been responsible for the current distribution of the ichthyofauna in the basins under consideration, as also proposed in previous studies with other species present in these basins (Vicari *et al.* 2005). On the other hand, the observed differences concerning C-banding evidence that one of these populations, more specifically the one from the Jaguariaíva river, is differentiated from the others regarding this character, possibly as a consequence of gene flow restriction between them throughout geological and evolutionary history.

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