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Chromosomal diversification of reef fishes from genus *Centropyge* (Perciformes, Pomacanthidae)

Paulo Roberto Antunes de Mello Affonso & Pedro Manoel Galetti Jr.*
*Departamento de Genética e Evolução, Universidade Federal de São Carlos, Via Washington Luís, Km 235, Caixa Postal 676, 13565-905 São Carlos SP, Brazil; *Author for correspondence (Phone: +55-16-260-8309; Fax: +55-16-260-8306; E-mail: galettip@power.ufscar.br)*

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Abstract

The genus *Centropyge* is remarkable for species richness, composing a highly specialized fish group amongst members from family Pomacanthidae. However, cytogenetical reports are nearly absent in these animals. New data are provided from karyotypical studies carried out on *Centropyge aurantonotus* from the Brazilian coast of the Atlantic Ocean and *C. ferrugatus* from the Philippines Sea of the Indo-Pacific Ocean. Both species present $2n = 48$ but karyotypes are differentiated by fundamental number. *C. aurantonotus* has a great number of biarmed chromosomes (4 m + 14 sm + 16 st + 4 a), while *C. ferrugatus* presents only acrocentric chromosomes. Single nucleolar organizer regions (NORs) are located at interstitial position of an acrocentric pair in *C. ferrugatus* and on short arms of a subtelocentric pair in *C. aurantonotus*, as confirmed by fluorescent *in situ* hybridization (FISH) with 18S rDNA probes. Heterochromatin is distributed over NOR and centromeric regions in both species, but additional GC-rich heterochromatic blocks on short arms of up to eight chromosomal pairs can be detected in *C. aurantonotus*. 5S rDNA segments were located interstitially on two chromosomal pairs in *C. ferrugatus* and on nine pairs in *C. aurantonotus*, mostly equivalent to heterochromatic blocks on short arms of biarmed chromosomes. *C. ferrugatus* can be considered a species in which basal chromosomal features proposed for modern Teleosteans were conserved. The derived karyotype pattern of *C. aurantonotus* seems to be determined by pericentric inversions and heterochromatin addition which probably determined the notorious dispersion of 5S rRNA (pseudo)genes. It is demonstrated that, even within a group generally characterized by cytogenetical homogeneity as the family Pomacanthidae, diversified karyotypes can be found.

Introduction

Pomacanthidae is a specialized fish family, composed by about nine genera and more than 80 species, all associated with coral reefs and widespread throughout the Atlantic, Indian and Pacific Oceans (Johnson & Gill, 1995).

The genus *Centropyge* is a very specious Pomacanthidae group (nearly 40 species), with recent descriptions of new species (Pyle, 1990; Pyle & Randall, 1993). Representatives of this genus are popularly known as pigmy angelfishes, as a reference to their small size when compared to the correlated species. This feature and their beautiful color patterns make these fishes popular for ornamental aquarium (Hunziker, 1992). Furthermore, *Centropyge* species are some of most studied angelfishes under behavioral and reproductive focus, mainly concerned to the presence of protogynous hermaphroditism (Bauer & Bauer, 1981).

The only chromosomal report on *Centropyge* (Arai & Inoue, 1975), characterized the species *C. vrolikii*, identifying 48 acrocentric chromosomes. Such a karyotype is usually associated to the basal pattern of Perciformes, apparently highly conserved within marine species, including Pomacanthidae (Galetti, Aguilar & Molina, 2000; Affonso et al., 2001).

Nevertheless, faced to species diversity of the genus *Centropyge*, high genetic diversity (Chung & Woo, 1998) and the scarcity of cytogenetical information, it seems inappropriate to assume that conserved chromosomal features are characteristic for this Pomacanthidae group.

Thus, conventional and banding cytogenetical procedures, including fluorescent *in situ* hybridization (FISH) with rDNA probes, were carried out on two species of *Centropyge* from distinct Oceans, in order to evaluate the karyotype structure of the genus and family Pomacanthidae, as well. These species are *Centropyge aurantonotus* from the Western coast of the South Atlantic and *C. ferrugatus* from the Philippines Sea at Indo-Pacific Ocean.

Material and methods

Twelve adult individuals of *C. aurantonotus* were collected along the Atlantic shore of two municipalities on the Southeastern coast of Brazil (Marataízes/ES and Arraial do Cabo/RJ). Two specimens of *C. ferrugatus* from Philippines Sea at Indo-Pacific Ocean were obtained from ornamental fish collectors.

Twenty-four hours prior to cytogenetical preparations, the animals were inoculated with a suspension of biological yeast and kept in an aquarium. The *in vitro* obtaining of mitotic chromosomes from kidney, liver, spleen and gill tissues of specimens followed the procedure elsewhere described (Foresti, Oliveira & Almeida-Toledo, 1993). Chromosomal morphology was based on arm ratio (Levan, Fredga & Sandberg, 1964).

The nucleolar organizer regions (NORs) were detected by silver nitrate staining (Howell & Black, 1980), and by chromomycin A_3 (CMA_3) fluorescent staining (Schweizer, 1976). Heterochromatin was detected by barium hydroxide C-banding (Sumner, 1972).

The chromosomal localization of 18S and 5S rRNA genes was determined by fluorescent *in situ* hybridization (FISH), according to the method described by Pinkel, Straume and Gray (1986), using 18S rDNA probes from *Parodon hilarii* (Vicente, Jesus & Moreira-Filho, 2001) and 5S rDNA probes from *Leporinus elongatus* (Martins & Galetti, 1999).

Results

Both analyzed species were characterized by a diploid number of 48 chromosomes, invariably.

In *C. ferrugatus*, all chromosomes are acrocentric (Figure 1a). Occasionally, a secondary constriction at interstitial region on long arms of one large chromosomal pair could be observed. Analysis of somatic metaphases in this species allowed us to detect some chromosomes in association by their centromeric region (Figure 2).

C. aurantonotus presented a great number of biarmed chromosomes, being the karyotype composed by four metacentric, 14 submetacentric, 26 subtelocentric and four acrocentric chromosomes (Figure 1b).

Commonly, a secondary constriction on the short arms of 18th pair was present. The size

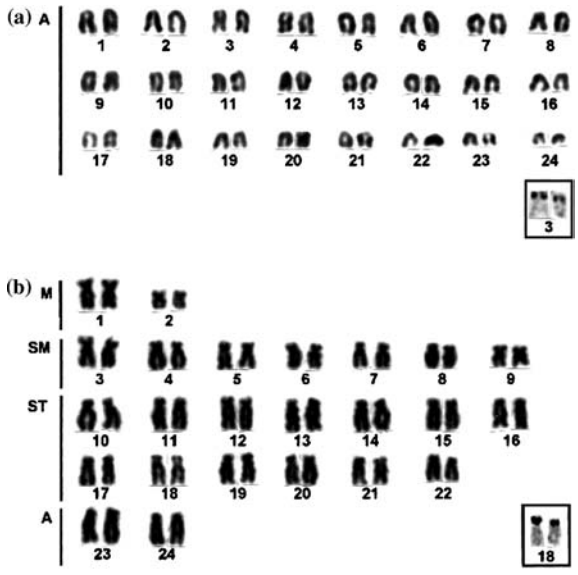

Figure 1. Karyotypes of *C. ferrugatus* ($2n = 48$) (a) and *C. aurantonotus* ($2n = 48$) (b). On inset, the NOR-bearing chromosomal pair after silver nitrate staining.

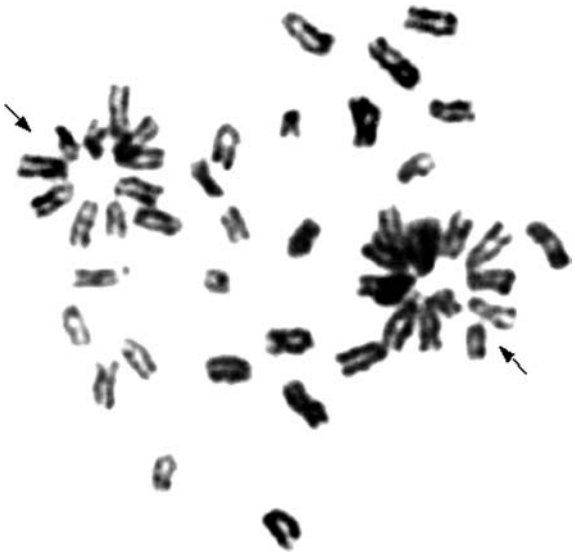

Figure 2. Somatic metaphase of *C. ferrugatus* showing some chromosomes associated by centromeres, with radial disposition (arrows).

variation of such constrictions resulted in heteromorphism between homologues.

For both species, a single NOR-bearing chromosome pair was identified after silver nitrate staining, but NOR location was different for each of them. In *C. ferrugatus*, positive marks were situated on interstitial region of an acrocentric pair (arranged as the third one of karyotype) (Figure 1a). In *C. aurantonotus*, active rDNA sites were located on short arms of the 18th chromosomal pair, coincident to secondary constrictions (Figure 1b).

By C-banding, significant differences were revealed between both species. In *C. ferrugatus*, heterochromatic portions were restricted to NORs and centromeric regions, representing a small amount of heterochromatin (Figure 3a).

Besides the centromeric marks, conspicuous heterochromatic blocks could be visualized in *C. aurantonotus*, occupying, entirely, the short arms of chromosomal pairs 2 (metacentric), 7, 9 (submetacentric), 12, 17, 18 (NORs), 20, 21, and 22 (subtelocentric) (Figure 3b).

By GC-specific fluorochrome staining (CMA_3), one marked chromosomal pair in *C. ferrugatus* was detected corresponding to the NOR-bearing chromosomes (Figure 4a).

On the other hand, in *C. aurantonotus* positive marks were detected on the 18th pair, similar to silver staining pattern, and on short arms of other eight chromosomal pairs of meta-, submeta- and subtelocentric types, although less evident than that on NORs (Figure 4b).

FISH with 18S rDNA probes confirmed the previous results obtained by silver nitrate, by the identification of a single NOR-bearing chromosome pair in both species (Figure 5(a) and (b)). FISH experiments revealed a structural heteromorphism of NOR size between homologous,

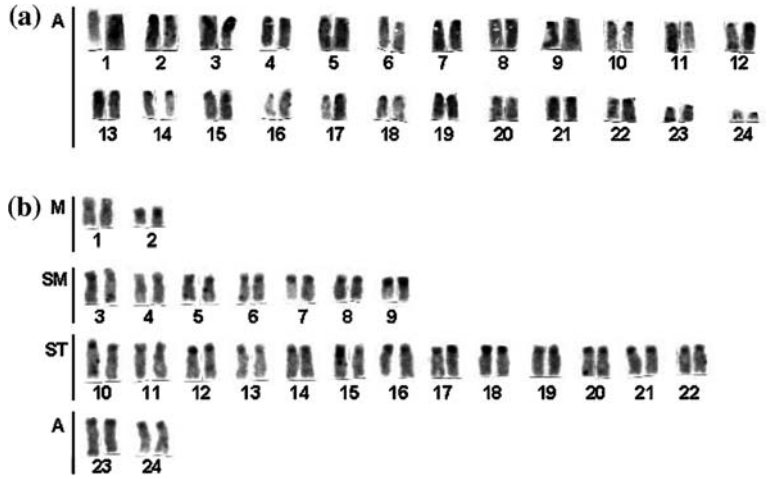

Figure 3. C-banded karyotypes. (a) *C. ferrugatus*, where heterochromatin distribution is restricted to NORs (third pair) and centromeric regions; (b) *C. aurantonotus*, with presence of constitutive heterochromatin on centromeres, and, conspicuously, on short arms of chromosomal pairs 2, 7, 9, 12, 17, 18 (NORs), 20, 21 and 22.

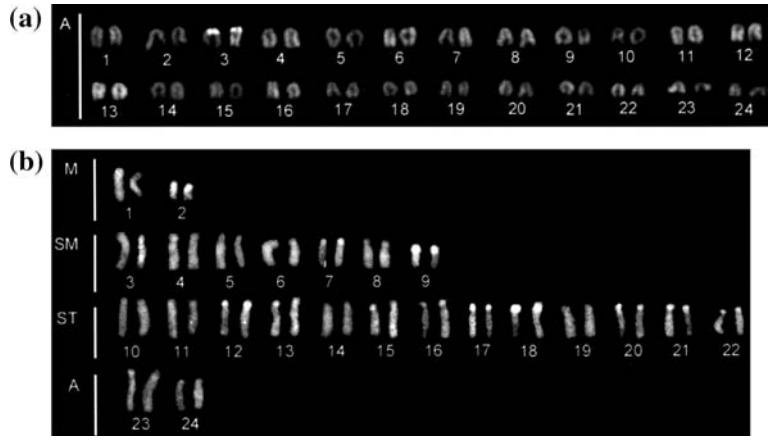

Figure 4. CMA_3 fluorescent stained karyotypes. (a) *C. ferrugatus* showing positive signals on NORs (third pair); (b) *C. aurantonotus* with positive signals on NOR-bearing pair (18) and, additionally, on short arms of several other biarmed chromosomes, comprising a total of 18 positively signed chromosomes.

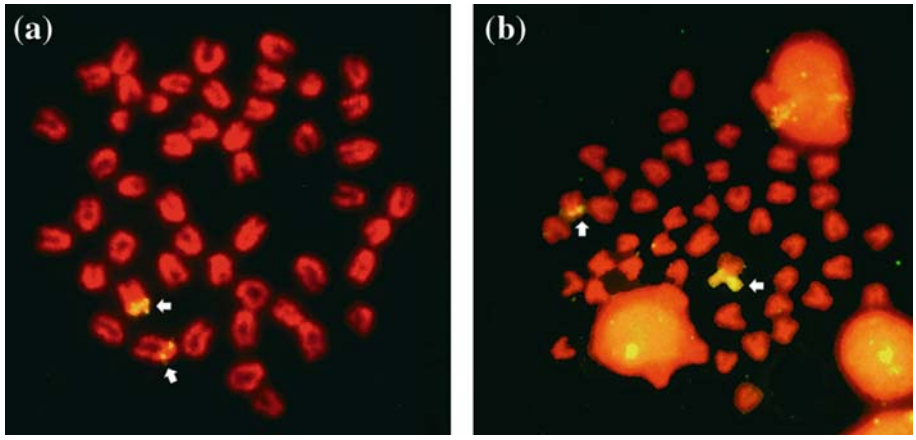

Figure 5. Metaphases of *C. ferrugatus* (a) and *C. aurantonotus* (b) after FISH with 18S rDNA probe, revealing one NOR-bearing pair, as indicated by arrows.

specially detectable in *C. aurantonotus* (Figure 5b). When 5S rDNA probes were employed, a remarkably distinct distribution pattern of 5S rRNA genes was observed between both *Centropyge* species. While *C. ferrugatus* presented two chromosomal pairs bearing 5S rDNA on interstitial position (Figure 6a), *C. aurantonotus* presented up to 18 positive signals, mostly distributed over heterochromatic blocks on short arms of several biarmed chromosomes and on interstitial position at long arms in one pair (Figure 6b). At least, for *C. ferrugatus*, 5S and 18S rDNA clusters are not syntenic, as it was possible to detect the NOR-bearing pair by the presence of NOR-associated heterochromatin, strongly stained with iodide propidium and negatively marked by 5S rDNA probe (Figure 6a). Sequential staining in *C. aurantonotus* failed and it was not possible to verify if 5S and 18S rDNA clusters are syntenic.

Discussion

The presence of 48 acrocentric chromosomes in Perciformes karyotypes is considered a basal condition for this group (Galetti, Aguilar & Molina,

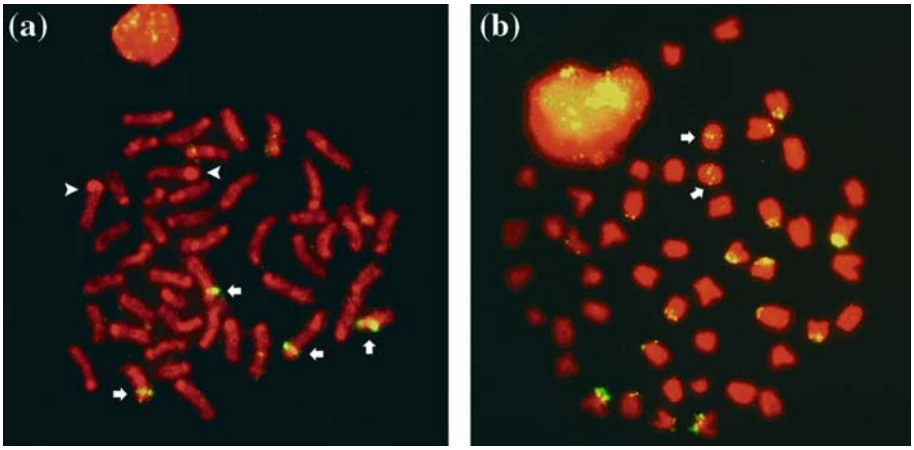

Figure 6. Metaphases of *C. ferrugatus* (a) and *C. aurantonotus* (b) after FISH with 5S rDNA probe, showing four positive signals in the former species (arrows) and up to 18 marks in *C. aurantonotus* (the arrows indicate the pair bearing interstitial signals). Note that 5S rRNA genes are not located at the NOR-bearing pair in *C. ferrugatus*, whose NOR-associated heterochromatin is strongly stained (indicated by arrowheads).

2000). Previous studies in Pomacanthidae indicated that all analyzed species ($n = 6$) present the typical karyotype of Perciformes or close to it (Arai & Inoue, 1975; Affonso et al., 2001; Affonso et al., 2002).

Single NORs at interstitial position and the presence of heterochromatic blocks associated to centromeres and NORs have also been correlated to ancestry in some Perciformes families, including Pomacanthidae (Affonso et al., 2002). More recently, FISH experiments using 18S rDNA and 5S rDNA probes have indicated that the presence of two chromosomal pairs bearing interstitial 5S rDNA sites may be a rule amongst fish species (Martins & Galetti, 2001; Sola et al., 2003), as well as the presence of distinct chromosomes carrying 45S and 5S rDNA clusters (Deiana et al., 2000).

By this way, the present cytogenetical data in *C. ferrugatus*, which corresponds exactly to the pattern commented above, identifies this species as another example of Perciformes with a highly conserved karyotypical structure. This evidence, in contrast to the specialization status of Pomacanthidae, could indicate that speciation process in this family was not derived or followed by karyotypical modifications (Molina, Maia-Lima & Affonso, 2002).

On the other hand, for *C. aurantonotus*, it is suggested a completely distinct karyoevolutionary pathway. Firstly, despite the maintenance of the diploid number ($2n = 48$), several biarmed chromosomes were detected in *C. aurantonotus* (FN = 92), diverging from the primitive fundamental number (FN = 48) proposed for Perciformes. This karyotype would be determined by pericentric inversions, a common rearrangement, responsible for most of karyotypical diversification observed within this fish group. (Galetti, Aguilar & Molina, 2000).

Numerically, the NOR pattern of *C. aurantonotus* agrees with the basal model for vertebrates, but its location (short arms of a subtelocentric pair) could be considered derived for family Pomacanthidae, probably resulted from pericentric inversion on an original acrocentric pair with interstitial NORs (Affonso et al., 2002). Also, a remarkable size heteromorphism between homologues was detected, either by silver nitrate staining or FISH with 18S rDNA probe, demonstrating that it comprises functional and structural aspects.

The application of C-banding procedure was of fundamental importance on karyotypical analysis of *C. aurantonotus*. Besides those C-positive marks at NORs and centromeric region, eight biarmed chromosome pairs presented conspicuous heterochromatic blocks on their short arms. This evidence suggests that heterochromatin segments were added to formerly acrocentric chromosomes. Alike the NOR site, these heterochromatic blocks on short arms of several chromosome pairs were also CMA_3^+, but lacking 45S rDNA clusters as confirmed by FISH.

The heterogeneity of heterochromatic segments related to compositional differences is not unusual, including examples in fish (Souza, Moreira-Filho & Galetti, 1996), but poorly described in tropical marine species. As all additional blocks presented the same composition, it is suggested that they share a common origin. It is possible that a primary segment of GC-rich heterochromatin have been dispersed throughout the chromosome complement of *C. aurantonotus*. After that, these segments could be amplified or accumulated by unequal exchanges, transpositions and/or regional duplication, similarly to the model proposed by Margarido and Galetti (2000) in the fish *Leporinus desmotes* (Characiformes). A non-random chromosomal arrangement during interphase could favor to attachment of chromosomal regions. This may facilitate the heterochromatin dispersion to equilocal sites from one chromosome to another (see Schweizer, Loidl & Hamilton, 1987). The frequent centromeric association of acrocentric chromosomes in Perciformes (Molina & Galetti, 2002), as seen in *C. ferrugatus*, provides a theoretical basis for the origin of heterochromatin dispersion occurred in *C. aurantonotus*.

The results provided by FISH using 5S rDNA probe also evidenced the role of heterochromatin dispersion in *C. aurantonotus*. The presence of several 5S rDNA sites overlapping GC-rich heterochromatic segments is another indication that this heterochromatin shares a common origin. Besides the interstitial 5S rDNA cluster, usually detected in fish species, another 5S rDNA cluster, interspersed with such heterochromatin, was equally distributed along the karyotype, determining one of the highest numbers of 5S rDNA cistrons observed in fishes so far. The linkage between GC-rich heterochromatin and rDNA dispersal was previously detected in other fish species, as *Leporinus desmotes*, but involving 45S clusters (Margarido & Galetti, 2000), whereas the association of CMA_3 positive marks and 5S rDNA sites was reported only in another Perciform species, *Micropterus salmoides* (Deiana et al., 2000). The effects of this increased distribution of 5S rRNA genes is unknown, but some mechanism of gene regulation should be acting to avoid an over expression. Alternatively, it is plausible to assume that some, if not most, of the positive signals detected by FISH with 5S rDNA probe, correspond to pseudogenes, displaying a very similar sequence to that of 5S rRNA genes, but inactive. The identification of pseudogenes was previously reported by other authors in some animal groups (Leah et al., 1990; Martins et al., 2002). The fact that these widespread positive signals are located on heterochromatic regions suggests the presence of pseudogenes, as heterochromatin is normally related to gene inactivation. Nevertheless, to confirm such hypothesis, sequencing and gene expression analyses should be performed.

Finally, the present data allow for the identification of a novel chromosomal diversification in Pomacanthidae. It is possible that cytogenetical differences between *C. aurantonotus* and *C. ferrugatus* would be a direct effect of distinct evolutionary pathways. It is postulated that sea level decreases during glacial periods at Atlantic Ocean determined a bottleneck in several fish populations, even those characterized by great dispersal (Goodbred & Graves, 1996). Such processes could have favored to a faster establishment of a new karyotypical pattern by drift effects in *C. aurantonotus*. Similar findings were reported in reef fishes of the genus *Chromis* (Pomacentridae) (Molina & Galetti, 2002).

It is also demonstrated that, as long as the cytogenetical surveys are improved on fishes from coral reefs, it is possible to detect differentiated levels of chromosomal diversification, and further studies in other representatives from this realm could contribute to our knowledge of cytogenetical patterns of the family Pomacanthidae.

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References

Affonso, P.R.A.M., W. Guedes, E. Pauls & P.M. Galetti Jr., 2001. Cytogenetic analysis of coral reef fishes from Brazil (families Pomacanthidae and Chaetodontidae). Cytologia 66: 379–384.

Affonso, P.R.A.M., W. Guedes, E. Pauls & P.M. Galetti Jr., 2002. Close karyotypical relationship between two species of marine angelfishes from South Atlantic: *Pomacanthus*

arcuatus and *P. paru* (Perciformes, Pomacanthidae). Caryologia 55: 323–329.

Arai, R. & M. Inoue, 1975. Chromosomes of nine species of Chaetodontidae and one species of Scorpidae from Japan. Bull. Natl. Sci. Museum Tokyo, Ser. A (Zoology) 1: 217–224.

Bauer, J.A. & S.E. Bauer, 1981. Reproductive biology of pigmy angelfishes of the genus *Centropyge* (Pomacanthidae). Bull. Mar. Sci. 31(3): 495–513.

Chung, K.C. & N.Y.S. Woo, 1998. Phylogenetic relationships of the Pomacanthidae (Pisces: Teleostei) inferred from allozyme variation. J. Zool. 246: 215–231.

Deiana, A. M., A. Cau, S. Salvadori, E. Coluccia, R. Camas, A. Milia & J. Tagliavini, 2000. Major and 5S ribosomal sequences of the largemouth bass *Micropterus salmoides* (Perciformes, Centrarchidae) are localized in GC-rich regions of the genome. Chrom. Res. 8: 213–218.

Foresti, F., C. Oliveira & L.F. Almeida-Toledo, 1993. A method for chromosome preparations from large specimens of fish using *in vitro* short treatment with colchicine. Experientia 49: 810–813.

Galetti Jr. P.M., C.T. Aguilar & W. Molina, 2000. An overview of marine fish cytogenetics. Hydrobiologia 420: 55–62.

Goodbred, C.O. & J.E. Graves, 1996. Genetic relationships among geographically isolated populations of bluefish (*Pomatomus saltatrix*). Mar. Freshwater Res. 47: 347–355.

Howell, W.M. & D.A. Black, 1980. Controlled silver staining of nucleolus organizer region with protective colloidal developer: a 1-step method. Experientia 36: 1014–1015.

Hunziker, R., 1992. The littlest angels. Tropical Fish Hobbyist 41: 80–92.

Johnson, G.D. & A.C. Gill, 1995. Perches and their allies, pp. 181–196 in Encyclopedia of Fishes, edited by Paxton, J.R. & W.N. Eschemeyer. Academic Press Inc., San Diego.

Leah, R., S. Frederiksen, J. Engberg & P.D. Sorensen, 1990. Nucleotide sequence of a mouse 5S rRNA variant gene. Nucl. Acids Res. 18: 7441–7441.

Levan, A., K. Fredga & A.A. Sandberg, 1964. Nomenclature for centromeric position on chromosomes. Hereditas 52: 201–220.

Margarido, V.P. & P.M. Galetti Jr., 2000. Amplification of a GC-rich heterochromatin in the freshwater fish *Leporinus desmotes* (Characiformes, Anostomidae). Geneti. Mol. Biol. 23(3): 569–573.

Martins, C. & P.M. Galetti Jr., 1999. Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). Chrom. Res. 7: 363–367.

Martins, C. & P.M. Galetti Jr., 2001. Two 5S rDNA arrays in Neotropical fish species: is it a general rule for fishes? Genetica 111: 439–446.

Martins, C., A.P. Wasko, C. Oliveira, F. Porto-Foresti, P.P. Parise-Maltempi, J.M. Wright & F. Foresti, 2002. Dynamics of 5S rDNA in the tilapia (*Oreochromis niloticus*) genome: repeat units, inverted sequences, pseudogenes and chromosome loci. Cytogenet. Genome Res. 98: 78–85.

Molina, W.F. & P.M. Galetti Jr., 2002. Robertsonian rearrangements in the reef fish *Chromis* (Perciformes, Pomacentridae) involving chromosomes bearing 5S rRNA genes. Genet. Mol. Biol. 24(4): 373–377.

Molina, W.F., F.A. Maia-Lima & P.R.A.M. Affonso, 2002. Divergence between karyotypical pattern and speciation events in Serranidae fish (Perciformes). Caryologia 55(4): 299–305.

Pinkel, D., T. Straume & J.W. Gray, 1986. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc. Natl. Acad. Sci. 83: 2934–2938.

Pyle, R.L., 1990. *Centropyge debelius*, a new species of angelfish (Teleostei: Pomacanthidae) from Mauritius and Reunion. Rev. Fr. Aquariol 17: 53–56.

Pyle, R.L. & J.E. Randall, 1993. A new species of *Centropyge* from Cook Islands, with a redescription of *Centropyge boylei*. Rev. Fr. Aquariol 19: 115–124.

Schweizer, D., 1976. Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 92: 143–148.

Schweizer, D., J. Loidl & B. Hamilton, 1987. Heterochromatin and the phenomenon of chromosome banding, in Results and Problems in Cell Differentiation 14: Structure and Function of Eukaryotic Chromosomes, edited by Hennig, W. Springer-Verlag, Berlin.

Sola, L., A.R. Rossi, F. Annesi & E. Gornung, 2003. Cytogenetic studies in *Sparus auratus* (Pisces, Perciformes): molecular organization of 5S rDNA and chromosomal mapping of 5S and 45S ribosomal genes and of telomeric repeats. Hereditas 139: 232–236.

Souza, I.L., O. Moreira-Filho & P.M. Galetti Jr., 1996. Heterochromatin differentiation in the characid fish *Astyanax scapripinnis*. Brazilian J. Genet. 19(3): 405–410.

Sumner, A., 1972. A single technique for demonstrating centromeric heterochromatin. Exp. Cell Res. 75: 304–306.

Vicente, V.E., C.M. Jesus & O. Moreira-Filho, 2001. Chromosomal localization of 5S and 18S rRNA genes in three *Parodon* species (Pisces, Parodontidae). Caryologia 54(4): 365–359.