

# Heterochromatin differentiation in the characid fish *Astyanax scabripinnis*

Issakar Lima Souza, Orlando Moreira-Filho and Pedro Manoel Galetti Junior

## ABSTRACT

Chromosomes of a small characid fish, *Astyanax scabripinnis*, were submitted to several types of chromosome banding. Three different types of C-band positive heterochromatin were detected: *i*) minute centromeric and pericentromeric blocks in most of the chromosomes in the complement; *ii*) clearly visible blocks on the short arm of pair No. 18, corresponding to Ag-NOR sites, and conspicuous fluorescent chromomycin A<sub>3</sub> (CMA<sub>3</sub><sup>+</sup>) and mitramycin A (MM<sup>+</sup>), and *iii*) heterochromatic regions strongly stained with Giemsa after C-banding, which were distamycin A/4'-6-diamidine-2-phenylindole (DA/DAPI) and MM/DAPI, which showed no differentiation by silver nitrate, CMA<sub>3</sub>, MM, or DAPI without counterstaining. The equilocal distribution of these DA/DAPI heterochromatins suggests a common origin for these chromosome segments in *A. scabripinnis*.

## INTRODUCTION

Fish cytogenetics has been mainly limited to karyotyping and some routine chromosome bandings such as C-banding for heterochromatin identification and silver nitrate staining for the detection of nucleolar organizer regions (Ag-NORs). Much of this information has been interpreted according to an evolutionary approach, representing a valuable tool for the systematic and evolutionary understanding of the different fish groups. Recently, higher-resolution methods such as replication bands by 5-BrdU incorporation (Almeida-Toledo *et al.*, 1988; Venere, 1991; Hellmer *et al.*, 1991; Vicente, 1994), G-banding (Gold and Li, 1991; Bertollo, 1994), fluorescent stains (Mayr *et al.*, 1985, 1990), restriction enzymes banding (Lloyd and Thorgaard, 1988; Sánchez *et al.*, 1991; Hartley, 1991; Maistro, 1996) and *in situ* hybridization

with non-isotopic probes (Haaf *et al.*, 1993; Pendás *et al.*, 1994; Galetti Jr. *et al.*, 1995) have made important contributions to the understanding of the compositional and structural organization of fish chromosomes.

DNA-binding fluorochromes basically belong to two categories: those that preferentially bind to GC-rich sequences, the most common of them being chromomycin A<sub>3</sub> (CMA<sub>3</sub>) and mitramycin A (MM), and AT-specific ones such as quinacrine mustard, DAPI (4'-6-diamidine-2-phenylindole) and Hoechst 33258 (Verma and Babu, 1995). In lower vertebrates, especially fish and amphibians, the combined use of these fluorochromes can provide interesting information about heterochromatin types. A close relationship between Ag-NOR sites and the fluorescent bands produced by GC-specific stains has been repeatedly documented in these animals (Schmid, 1980, 1982; Mayr *et al.*, 1985; Amemiya and Gold, 1986; Ráb and Mayr, 1987; Schmid and Guttenbach, 1988; Phillips *et al.*, 1989; Sola *et al.*, 1992; Galetti Jr. and Rasch, 1993a,b; Galetti Jr. *et al.*, 1995). On the other hand, fluorochromes such as DAPI produce very few bands along fish chromosomes.

When combined with the counterstain distamycin A (DA) (Schweizer, 1976), they have been useful for the detection of sex chromosomes in a species of *Poecilia* (Haaf and Schmid, 1984) and for the visualization of some heteropycnoses coinciding with nucleoli (Mayr *et al.*, 1988). However, they often produce either negative bands, coinciding with CMA<sub>3</sub> positive bands, or uniformly stained chromosomes.

In the present paper, C-banding, Ag-NORs and several fluorochrome staining methods have detected different heterochromatins in the chromosomes of the characid fish *A. scabripinnis*.

## MATERIAL AND METHODS

The chromosomes of two specimens (female and male) of *A. scabripinnis* collected from the Canta Galo stream, Tietê river basin, Brazil, were subjected to different banding methods. Mitotic chromosomes were obtained from kidney cells as described in details by Bertollo *et al.* (1978) and Moreira-Filho and Bertollo (1991). Constitutive heterochromatin was identified with the barium hydroxide method (Sumner, 1972) and the nucleolar organizer regions were detected by silver nitrate staining (Howell and Black, 1980).

Fluorescent bands were obtained using the fluorochromes CMA<sub>3</sub>, MM and DAPI. Distamycin A (DA), an AT-specific non-fluorescent antibiotic, was used as counterstain for the three fluorochromes (Schweizer, 1976; Schmid, 1980). The DA/CMA<sub>3</sub> and DA/MM preparations were kept in the dark for at least 15 days before exposure to the UV light (Galetti Jr. and Rasch, 1993b) and were analyzed with an excitation filter in the blue zone (450-490 nm) and barrier filter 530 nm. DA/DAPI slides stored in the dark for one day were analyzed under an excitation filter UV 360-390 nm and barrier filter of 435 nm. For some DAPI preparations, instead of distamycin A, mitramycin A was used as counterstain. The chromosomes were measured and classified according to Levan *et al.* (1964).

## RESULTS

The *A. scabripinnis* specimens had a diploid number of  $2n = 48$ , with the karyotype consisting of three pairs of metacentric chromosomes (M), 11 pairs of submetacentrics (SM), four pairs of subtelocentrics (ST) and six pairs of acrocentrics (A), with a fundamental number FN = 84, concordant with a previous report by Souza *et al.* (1995).

The distribution of constitutive heterochromatin exhibited the following pattern: *i*) minute centromeric and pericentromeric blocks in most of the chromosomes in the complement (Figures 1a and 2b); *ii*) clearly visible positive blocks on the short arm of pair No. 18 (Figure 2b), corresponding to Ag-NOR sites (Figure 2a), and conspicuous fluorescent CMA<sub>3</sub><sup>+</sup> and MM<sup>+</sup> bands (represented by CMA<sub>3</sub> staining in Figure 2c); *iii*) large telomeric and subtelomeric C-banding positive blocks plus some interstitial ones along six chromosome pairs - one subtelocentric pair and five acrocentric pairs (Figure 1a). In this latter, these heterochromatic blocks were clearly shown as DA/DAPI negative (Figure 1b). When used without the counterstain distamycin A, DAPI showed no differentiation along the chromosomes (Figure 2d). The MM/DAPI combination revealed broad MM/DAPI bands coinciding with DA/DAPI bands.

## DISCUSSION

Three different types of heterochromatin with different characteristics after banding procedures were found to be present in the genome of *A. scabripinnis*. The centromeric and pericentromeric heterochromatins present in most chromosomes were not highlighted by GC- or AT-specific fluorochromes, indicating that these regions do not have clusters with GC- or AT-rich sequences. In this heterochromatin, GC and AT sequences may have an interspersed arrangement that does not permit differentiation by any of the fluorochromes utilized. This absence of differentiation of centromeric heterochromatin submitted to fluorochromes has been reported several times and it seems to be the rule, rather than the exception, among amphibians and fish (Schmid, 1980; Mayr *et al.*, 1985, 1988, 1990; Schmid and Guttenbach, 1988; Sola *et al.*, 1990; 1992).

On the other hand, the positive C-bands of pair No. 18 (Figure 2b), which were also stained by silver nitrate (Figure 2a), representing chromosome regions involved in nucleolar organization, were found to be CMA<sub>3</sub><sup>+</sup> (Figure 2c) and DA/DAPI<sup>+</sup>, supporting a possible difference in the base ratio, with high predominance of GC bases. No other chromosome region was detected by CMA<sub>3</sub> or MM in this species. The coincidence of C<sup>+</sup>/Ag-NORs<sup>+</sup>/CMA<sub>3</sub><sup>+</sup>/MM<sup>+</sup> bands has been frequently reported in fish (Amemiya and Gold, 1986; Mayr *et al.*, 1985, 1988, 1990; Phillips and Hartley, 1988; Phillips *et al.*, 1989; Sola *et al.*, 1990, 1992; Galetti Jr. and Rasch, 1993a,b; Galetti Jr. *et al.*, 1995). In the Atlantic salmon, both ribosomal cistrons and their flanking het-

erochromatins were shown to be CMA<sub>3</sub><sup>+</sup> (Phillips and Hartley, 1988). Recently, Pendás *et al.* (1993) mapped the chromosomes of these salmon by fluorescent *in situ* hybridization (FISH) and observed that the ribosomal cistrons, in addition to being located in the secondary constriction, were also intertwined with heterochromatin portions adjacent to this constriction that were not detected by AgNO<sub>3</sub> staining. Among the probes used by Pendás *et al.* (1993), there were two that hybridized non-transcribed spacer rDNA, suggesting that the spacers, probably rich in GC, may be responsible for the coincidence of C<sup>+</sup>/Ag<sup>-</sup> NORs<sup>+</sup>/CMA<sub>3</sub><sup>+</sup>/MM<sup>+</sup> bands.

The DAPI staining, either combined or not with the counterstain distamycin, did not produce any highlighted region on the chromosomes of *A. scabripinnis*. Previous studies using DAPI staining have also reported a lack of fluorescent bands on the chromosomes of several other fish species (Schmid and Guttenbach, 1988; Souza and Moreira-Filho, 1995). It is strongly suggestive that AT sequences are unclustered in the fish genome. On the other hand, the occurrence of large blocks of DA/DAPI heterochromatin located in the terminal, subterminal and interstitial portions in six chromosome pairs suggests the presence of long stretches of AT-poor sequences in these regions. Contrary to what was expected, however, these same chromosome regions were not highlighted by GC-specific fluorochromes (CMA<sub>3</sub> or MM). Moreover, DAPI quenching was not observed when DAPI was used without any counterstain. Thus, this peculiar result might be due to the interaction between DAPI and the counterstains, instead of compositional characteristics of these DA/DAPI heterochromatins.

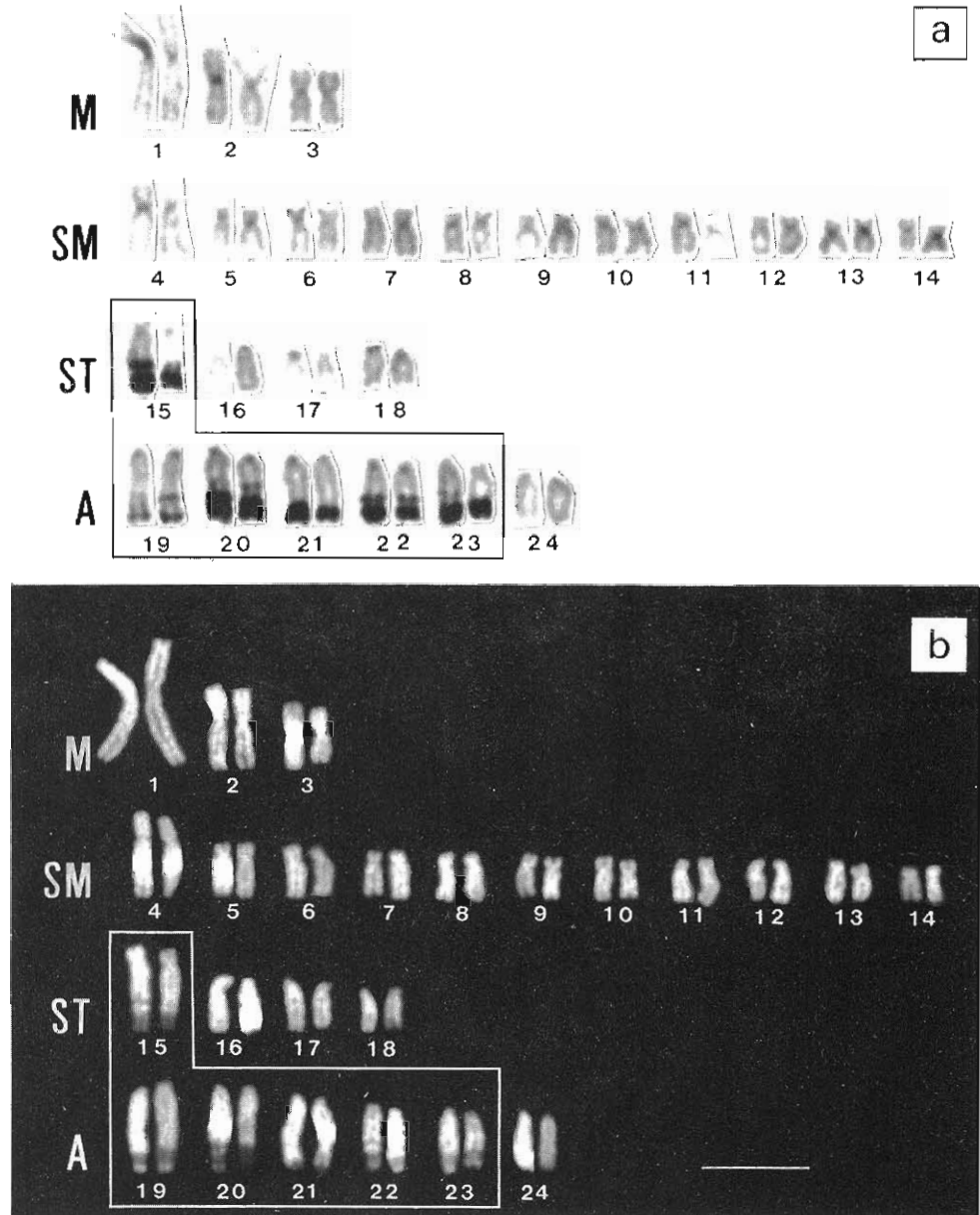


Figure 1 - C-banded (a) and DA/DAPI stained (b) karyotypes of *Astyanax scabripinnis* from Canta Galo brook (2n = 48). Chromosomes bearing large positive C-bands and DA/DAPI negative portions, in the insets. Bar = 5.0  $\mu$ m.

Two major classes of interaction between pairs of chromosome stains (primary stain and counterstain) responsible for the effects of fluorescent banding are basically transfer of energy excitation from one to the other, or competition for binding sites in the DNA segments along the chromosomes (Sahar and Latt, 1980). A conspicuous modification of DAPI banding has been reported in human chromosomes when slides are pre-stained with distamycin (Schweizer *et al.*, 1978). It is assumed that distamycin strongly binds to DNA with specific affinity for regions rich in AT bases, changing some physicochemical properties of DNA and also altering the geometric arrangement of these regions (Zim-

mer *et al.*, 1971). In the DA/DAPI negative heterochromatins of *A. scabripinnis*, distamycin may strongly compete with DAPI, reducing or preventing the access of this compound to DNA. However, the strong effect of DAPI quenching, also observed when mitramycin is used as counterstain, suggests that transfer of energy excitation might also be an important determinant of these negative bands.

The orderly positioning of these DA/DAPI heterochromatins on the terminal portions of the long arm of some chromosomes suggests a non-random distribution of these segments. The heterochromatin sequences may have been transferred to equilocal sites from one chromosome to another (Schweizer and Ehrendorfer, 1983; John *et al.*, 1985), facilitated by overlapping of most similar chromosome arms, related to a possible circular arrangement of non-homologous chromosomes in the interphase nucleus (Bennett, 1982). The similar behavior of these DA/DAPI heterochromatins when treated with fluorochromes, strongly supports a hypothesis of common origin. Amplifications of already existing sequences (Haaf and Schmid, 1984) or even substitutions or transformations of the chromatin (King, 1980), independently or in combination, may have resulted in the arrangement and composition of the heterochromatins found in *A. scabripinnis*.

## ACKNOWLEDGMENTS

The authors are grateful to Dr. Marcelo dos Santos Guerra for valuable suggestions, to Dr. Mirna Januária Leal Godinho for fluorescence microscopy facilities and to Adriana Medágia and Alois Copriva for technical assistance. This research was supported by Universidade Federal de São Carlos (UFSCar), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and

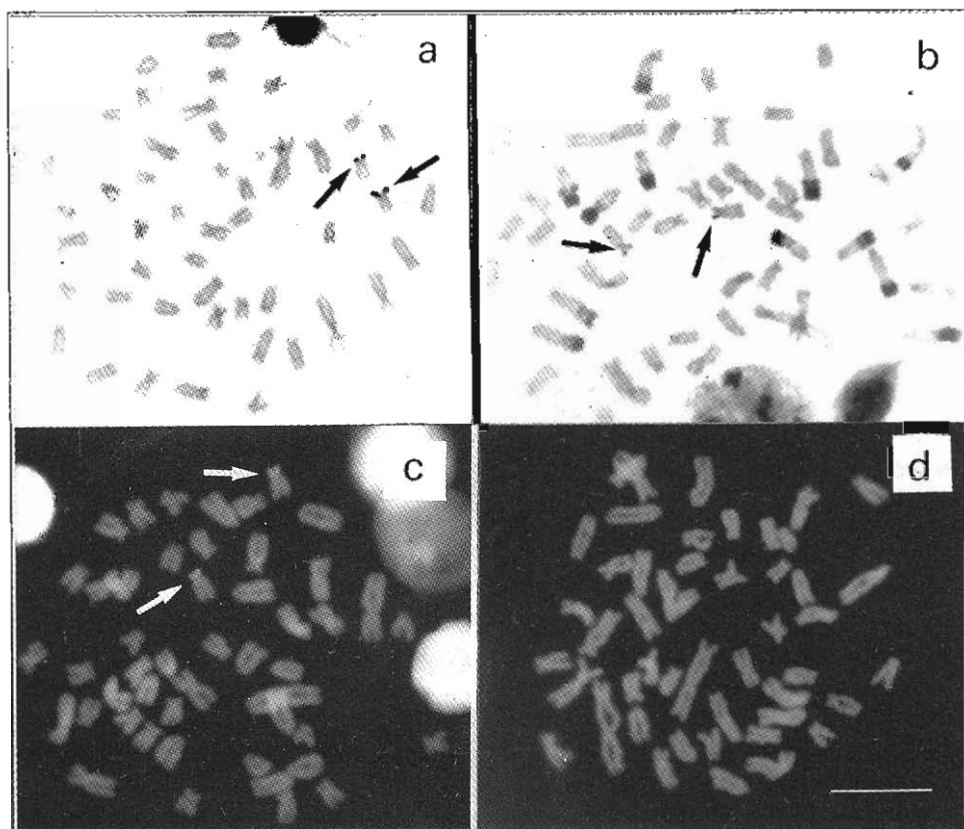


Figure 2 - Metaphases from several specimens of *Astyanax scabripinnis*. Silver nitrate stained (a), C-banded (b), DA/CMA stained (c), and DAPI stained, without counterstain (d). The arrows show chromosome pair 18. Bar = 5.0  $\mu$ m.

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Publication supported by FAPESP.

## RESUMO

Cromossomos mitóticos do pequeno peixe caracádeo *Astyanax scabripinnis* foram submetidos a diferentes tipos de bandeamento cromossômico e detectaram-se três tipos de heterocromatina banda C positiva: *i*) pequenas marcações centroméricas e pericentroméricas na maioria dos cromossomos do complemento; *ii*) bandas positivas claramente visíveis no braço curto do par 18, coincidindo com locais Ag-NORs<sup>+</sup> e evidentes cromomicina A<sub>3</sub> (CMA<sub>3</sub><sup>+</sup>) e mitramicina A (MM<sup>+</sup>); e *iii*) porções heterocromáticas fortemente coradas com Giemsa após o bandeamento C, e que mostraram-se distamicina A/4'-6-diamidina-2-fenilindol (DA/DAPI) e MM/DAPI, mas sem nenhuma diferenciação pelo AgNO<sub>3</sub>, CMA<sub>3</sub>, MM, ou o próprio DAPI sem contra-corante. Para este terceiro tipo de heterocromatina, são discutidas as relações de competição e/ou transferência de energia de excitação entre o DAPI e os contra-corantes. A distribuição equilocal dos blocos heterocromáticos DA/DAPI pode sugerir uma origem comum destes segmentos cromossômicos em *A. scabripinnis*.

## REFERENCES

- Almeida-Toledo, L.F., Viegas-Péquignot, E., Foresti, F., Toledo-Filho, S.A. and Dutrillaux, B.** (1988). BrdU replication patterns demonstrating chromosome homologies in two fish species, genus *Eigenmannia*. *Cytogenet. Cell Genet.* 48: 117-120.
- Amemiya, C.T. and Gold, R.** (1986). Chromomycin A<sub>3</sub> stains nucleolus organizer regions of fish chromosomes. *Copeia* 1: 226-231.
- Bennett, M.D.** (1982). The nucleotypic basis of the spatial ordering of chromosomes in eukaryotes and the implications of the order for genome evolution and phenotypic variation. In: *Genome Evolution* (Dover, G.A. and Flavell, R.B., eds.). Academic Press, London, pp. 239-261.
- Bertollo, L.A.C.** (1994). Bandamento G em cromossomos de peixes. V *Simpósio de Citogenética Evolutiva e Aplicada de Peixes Neotropicais*. Instituto de Biociências, UNESP, October 25-27, Botucatu, SP, Brazil, pp. 78 (Abstract).
- Bertollo, L.A.C., Takahashi, C.S. and Moreira-Filho, O.** (1978). Cytotaxonomic considerations on *Hoplias lacerdae* (Pisces, Erythrinidae). *Braz. J. Genet.* 1: 103-120.
- Burkholder, G.D. and Ducek, L.L.** (1982). The effect of chromosome banding techniques on the proteins of isolated chromosomes. *Chromosoma* 87: 425-435.
- Galetti Jr., P.M. and Rasch, E.M.** (1993a). NOR variability in diploid and triploid forms of the amazon molly *Poecilia formosa* as shown by silver nitrate and chromomycin A<sub>3</sub> staining. *Braz. J. Genet.* 16: 927-938.
- Galetti Jr., P.M. and Rasch, E.M.** (1993b). Chromosome studies in *Poecilia latipunctata* (Teleostei: Poeciliidae). *Ichthyol. Explor. Freshwaters* 4: 269-277.
- Galetti Jr., P.M., Mestriner, C.A., Monaco, P.J. and Rasch, E.M.** (1995). Post-zygotic modifications and intra- and interindividual nucleolar organizing regions variations in fish: report of a case involving *Leporinus friderici*. *Chrom. Res.* 3: 285-290.
- Gold, J.R. and Li, Y.C.** (1991). Trypsin G-banding of North American cyprinid chromosomes: Phylogenetic considerations, implications for fish chromosome structure, and chromosomal polymorphism. *Cytologia* 56: 199-208.
- Haaf, T. and Schmid, M.** (1984). An early stage of ZW/ZZ sex chromosome differentiation in *Poecilia sphenops* var. *melanistica* (Poeciliidae, Cyprinodontiformes). *Chromosoma* 89: 37-41.
- Haaf, T., Schmid, M., Steinlein, C., Galetti Jr., P.M. and Willard, H.F.** (1993). Organization and molecular cytogenetics of a satellite DNA family from *Hoplias malabaricus* (Pisces, Erythrinidae). *Chrom. Res.* 1: 77-86.
- Hartley, S.E.** (1991). C, Q and restriction enzyme banding of the chromosomes in brook trout (*Salvelinus fontinalis*) and Arctic charr (*Salvelinus alpinus*). *Hereditas* 114: 253-261.
- Hellmer, A., Voiculescu, I. and Schempp, W.** (1991). Replication banding in two cyprinid fishes. *Chromosoma* 100: 524-531.
- Holmquist, G.** (1979). The mechanism of C-banding: Depurination and  $\beta$ -elimination. *Chromosoma* 72: 203-224.
- Howell, W.M. and Black, D.A.** (1980). Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Experientia* 36: 1014-1015.
- Jack, E.M., Harrison, C.J., Allen, T.D. and Harris, R.** (1985). The structural basis for C-banding. A scanning electron microscopy study. *Chromosoma* 91: 363-368.
- John, B., King, M., Schweizer, D. and Mendelak, M.** (1985). Equilocality of heterochromatin distribution and heterochromatin heterogeneity in acridid grasshoppers. *Chromosoma* 91: 185-200.
- King, M.** (1980). C-banding studies on Australian hylid frogs: Secondary constriction structure and concept of euchromatin transformation. *Chromosoma* 80: 191-217.
- Levan, A., Fredga, K. and Sandberg, A.A.** (1964). Nomenclature for centromeric position on chromosomes. *Hereditas* 52: 201-220.
- Lloyd, M.A. and Thorgaard, G.H.** (1988). Restriction endonuclease banding of rainbow trout chromosomes. *Chromosoma* 96: 171-177.
- Maistro, E.L.** (1996). Caracterização morfológica e estrutural dos cromossomos supranumerários em peixes. Doctoral thesis, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, SP.
- Mayr, B., Ràb, P. and Kalat, M.** (1985). Localization of NORs and counterstain-enhanced fluorescence in *Perca fluviatilis* (Pisces, Percidae). *Genetica* 67: 51-56.
- Mayr, B., Kalat, M. and Ràb, P.** (1988). Heterochromatins and band karyotypes in three species of salmonids. *Theor. Appl. Genet.* 76: 45-53.
- Mayr, B., Kalat, M. and Ràb, P.** (1990). Sequential counterstain-enhanced fluorescence chromosome banding in the fish *Anguilla anguilla*. *Caryologia* 43: 277-281.
- Moreira-Filho, O. and Bertollo, L.A.C.** (1991). Extraction and use of the cephalic kidney for chromosome studies in small fish. *Braz. J. Genet.* 14: 1085-1090.
- Pathak, S. and Arrighi, F.E.** (1973). Loss of DNA following C-banding procedures. *Cytogenet. Cell Genet.* 12: 414-422.
- Pendás A.M., Morán, P. and García-Vázquez, E.** (1993). Ribosomal RNA genes are interspersed throughout a heterochromatic chromosome arm in Atlantic salmon. *Cytogenet. Cell Genet.* 63: 128-130.
- Pendás, A.M., Morán, P. and García-Vázquez, E.** (1994). Organization and chromosomal location of the major histone cluster in brown trout, Atlantic salmon and rainbow trout. *Chromosoma* 103: 147-152.
- Phillips, R.B. and Hartley, S.E.** (1988). Fluorescent banding patterns of the chromosomes of the genus *Salmo*. *Genome* 30: 193-197.
- Phillips, R.B., Pleyte, K.A. and Ihssen, P.E.** (1989). Patterns of chromosomal nucleolar organizer region (NOR) variation in fishes of the genus *Salvelinus*. *Copeia* 1989: 47-53.
- Ràb, P. and Mayr, B.** (1987). Chromosome banding studies in European esocoid fishes: localization of nucleolar organizer regions in *Umbra krameri* and *Esox lucius*. *Copeia* 1987: 1062-1067.
- Rocchi, A.** (1982). On the heterogeneity of heterochromatin. *Caryologia* 35: 169-189.

- Sahar, E. and Latt, S.A.** (1980). Energy transfer and binding competition between dyes used to enhance staining differentiation in metaphase chromosome. *Chromosoma* 79: 1-28.
- Sánchez, L., Martínez, P., Bonza, C. and Viñas, A.** (1991). Chromosomal heterochromatin differentiation in *Salmo trutta* with restriction enzymes. *Heredity* 66: 241-249.
- Schmid, M.** (1980). Chromosome banding in Amphibia. IV. Differentiation of GC- and AT-rich chromosome regions in Anura. *Chromosoma* 77: 83-103.
- Schmid, M.** (1982). Chromosome banding in Amphibia. VII. Analysis of the structure and variability of NORs in Anura. *Chromosoma* 87: 327-344.
- Schmid, M. and Guttenbach, M.** (1988). Evolutionary diversity of reverse R fluorescent chromosome bands in vertebrates. *Chromosoma* 97: 101-114.
- Schweizer, D.** (1976). Reverse fluorescent chromosome banding with chromomycin and DAPI. *Chromosoma* 58: 307-324.
- Schweizer, D. and Ehrendorfer, F.** (1983). Evolution of C-band patterns in Asteraceae - Antemidaeae. *Biol. Zentralbl.* 102: 637-655.
- Schweizer, D., Ambros, P. and Ardle, M.** (1978). Modification of DAPI banding on human chromosomes by prestaining with a DNA-binding oligopeptide antibiotic, distamycin A. *Exp. Cell Res.* 111: 327-332.
- Sola, L., Monaco, P.J. and Rasch, E.M.** (1990). Cytogenetics of bisexual/unisexual species of *Poecilia*. I. C-bands, Ag-NOR polymorphisms, and sex chromosomes in three populations of *Poecilia latipinna*. *Cytogenet. Cell Genet.* 53: 148-154.
- Sola, L., Rossi, A.R., Iaselli, V., Rasch, E.M. and Monaco, P.J.** (1992). Cytogenetics of bisexual/unisexual species of *Poecilia*. II. Analysis of heterochromatin and nucleolar organizer regions in *Poecilia mexicana* by C-banding and DAPI, quinacrine, chromomycin A<sub>3</sub> and silver staining. *Cytogenet. Cell Genet.* 60: 229-235.
- Souza, I.L. and Moreira-Filho, O.** (1995). Cytogenetic diversity in the *Astyanax scabripinnis* species complex (Pisces, Characidae). I. Allopatric distribution in a small stream. *Cytologia* 60: 1-11.
- Souza, I.L., Moreira-Filho, O. and Bertollo, L.A.C.** (1995). Cytogenetic diversity in the *Astyanax scabripinnis* species complex (Pisces, Characidae). II. Cytotypes living in sympatry. *Cytologia* 60: 273-281.
- Sumner, A.T.** (1972). A simple technique for demonstrating centromeric heterochromatin. *Exp. Cell Res.* 75: 304-306.
- Venere, P.C.** (1991). Citogenética comparativa de peixes da família Curimatidae (Characiformes). Master's thesis, Centro de Ciências Biológicas e da Saúde, Universidade Federal de São Carlos, São Carlos, SP.
- Verma, R.S. and Babu, A.** (1995). *Human Chromosomes: Principles and Techniques*. 2nd edn. McGraw-Hill Inc., New York.
- Vicente, V.E.** (1994). Estudos do cromossomo B em três populações de *Astyanax scabripinnis* (Pisces, Characidae). Master's thesis. Centro de Ciências Biológicas e da Saúde, Universidade Federal de São Carlos, São Carlos, SP.
- Zimmer, Ch, Reinert, K.E., Luck, G., Wähnert, U., Löber, G. and Thrum, H.** (1971). Interaction of the oligopeptide antibiotics netropsin and distamycin A with nucleic acids. *J. Mol. Biol.* 58: 329-348.

(Received January 8, 1996)