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Amplification of a GC-rich heterochromatin in the freshwater fish *Leporinus desmotes* (Characiformes, Anostomidae)

Vladimir Pavan Margarido[1] and Pedro Manoel Galetti Junior[2]

Abstract

This is the first description of the karyotype of *Leporinus desmotes*. The diploid female number was 2n = 54 meta- and submetacentric chromosomes. The nucleolar organizing regions (NORs) were studied by silver nitrate staining and rDNA fluorescence *in situ* hybridization (FISH) and were found to be located in the telomeric region of the long arm of the 9th pair. C-banding revealed centromeric and telomeric heterochromatin segments in most chromosomes. Intercalar blocks of heterochromatin were observed in the long arm of six chromosome pairs. Besides a NOR-adjacent heterochromatin, all of the intercalar heterochromatic segments were brightly fluorescent by mithramycin staining. These data suggest that a unique amplification of a primordial GC-rich heterochromatin, probably NOR-associated, may have taken place in the karyotype diversification of this *Leporinus* species.

INTRODUCTION

The heterochromatin has been reported to serve as an important source of karyotype diversification within and between groups of fish (Ojima and Ueda, 1979; Mayr *et al.*, 1985). The heterochromatin has been described primarily in the centromeric and telomeric areas in most neotropical fish species, but it can be much more abundant in several genera or species (Galetti *et al.*, 1991; Souza *et al*., 1996; Margarido and Galetti, 1996).

Since the C-banding was described as a method for heterochromatin identification along the chromosomes (Sumner, 1972), additional techniques have been used for investigating this particular chromosome segment (for review, see Sumner, 1990). Some fluorochromes showing base specific DNA-binding properties have revealed qualitative differences in the heterochromatin among the chromosome complement of *Astyanax scabripinnis* (Souza *et al*., 1996) and some *Leporinus* species (Molina *et al.*, 1998). Chromomycin A_3 and mithramycin A are GC-rich DNA sequence specific fluorochromes that often promote enhancement of the nucleolar organizing regions (NORs) in fish (Mayr *et al.*, 1985; Amemiya and Gold, 1986) and other lower vertebrates (Schmid and Guttenbach, 1988). However, some heterochromatin segments, which are non-related to NOR sites, can also exhibit fluorescence and brighter signals after GC-specific fluorochrome staining (Artoni *et al.*, 1999).

The heterochromatin has played an important role in the chromosomal diversification of the family Anostomidae (Galetti *et al.*, 1991), including that of sex chromosome differentiation in the genus *Leporinus* (Galetti and Foresti, 1986). Most part of this heterochromatin, however, has shown no differential staining with base-specific fluorochromes. These results suggest an absence of large AT- or GC-rich clusters in these fishes, except for those that are NOR-associated (Galetti *et al.*, 1995) and for the heterochromatic portion of the sex chromosomes observed only in some *Leporinus* species (Koehler *et al.*, 1997; Molina *et al.*, 1998).

In the present study, we report that C-banding and mithramycin staining revealed large amounts of an intercalar GC-rich heterochromatin in the karyotype of *Leporinus desmotes*, which could have arisen by amplification of a primordial NOR-associated heterochromatin. Additionally, silver nitrate staining and fluorescent *in situ* hybridization with 18S rDNA were used to investigate the NOR-heterochromatin relationship.

MATERIAL AND METHODS

Chromosome preparation and banding methods

Metaphases were obtained from cephalic kidney cells of two *Leporinus desmotes* females, collected in the Tocantins River (Porto Nacional, TO), according to the methods of Bertollo *et al.* (1978). Silver nitrate staining was used to detect AgNOR sites (Howell and Black, 1980). C-banding was visualized by using barium hydroxide (Sumner, 1972) and mithramycin A staining, using distamycin A counterstaining to investigate MM-banding pattern (Schmid, 1980).

[1]*Departamento de Ciências Biológicas, Universidade Estadual do Oeste do Paraná, Rua Universitária, 2069, 85814-110 Cascavel, PR, Brasil. Send correspondence to V.P.M. E-mail: vpmargarido@unioeste.br*
[2]*Departamento de Genética e Evolução, Universidade Federal de São Carlos, Via Washington Luís, km 235, 13565-905 São Carlos, SP, Brasil. E-mail: galettip@power.ufscar.br*

PCR and rDNA probe labeling

Genomic DNA was isolated from liver tissue as described by Sambrook *et al.* (1989). DNA samples were used for PCR amplification with a set of 18S rDNA primers as described by Hizume (1994). PCR products were analyzed in agarose gels, and a fragment of approximately 1.8 kb (corresponding to the 18S rDNA region) was purified using Pharmacia Biotech Bandprep eluation kit. An 18S rDNA probe was labeled by nick translation with biotin-7-dUTP (Roche) according to the manufacturer's instructions.

In situ hybridization and signal detection

Fluorescent *in situ* hybridization (FISH) was performed according to the method of Pinkel *et al.* (1986). The rDNA probe was detected by avidin-N-fluorescein isothiocyanate (FITC) conjugate, and the signal was enhanced by using biotinilated anti-avidin goat antibodies following a second round of the avidin-FITC detection. Chromosomes were counterstained with propidium iodide.

RESULTS

Karyotyping and chromosome banding analysis

Leporinus desmotes showed a karyotype of 2n = 54 composed of meta- and submetacentric chromosomes (Figure 1A). A large heterozygotic secondary constriction was often detected in the end of the long arm of the chromosome pair 9. C-banding revealed heterochromatic blocks in the centromeres and telomeres of most chromosomes. In addition, intercalar heterochromatin blocks were detected in the long arm of the chromosome pairs 2, 5, 6, 9, 10 and 11 (Figure 1B). In the pairs 2 and 6 the intercalar heterochromatin blocks appear duplicated. AgNORs were detected in the end of the long arm of a median-size metacentric, corresponding to pair 9 (Figure 1B). AgNOR homologous sites exhibited size polymorphism in all 30 metaphases analyzed, corroborating the secondary constriction heteromorphism. A large heterochromatin segment was detected adjacent to this secondary constriction. Mithramycin staining revealed a bright MM fluorescent

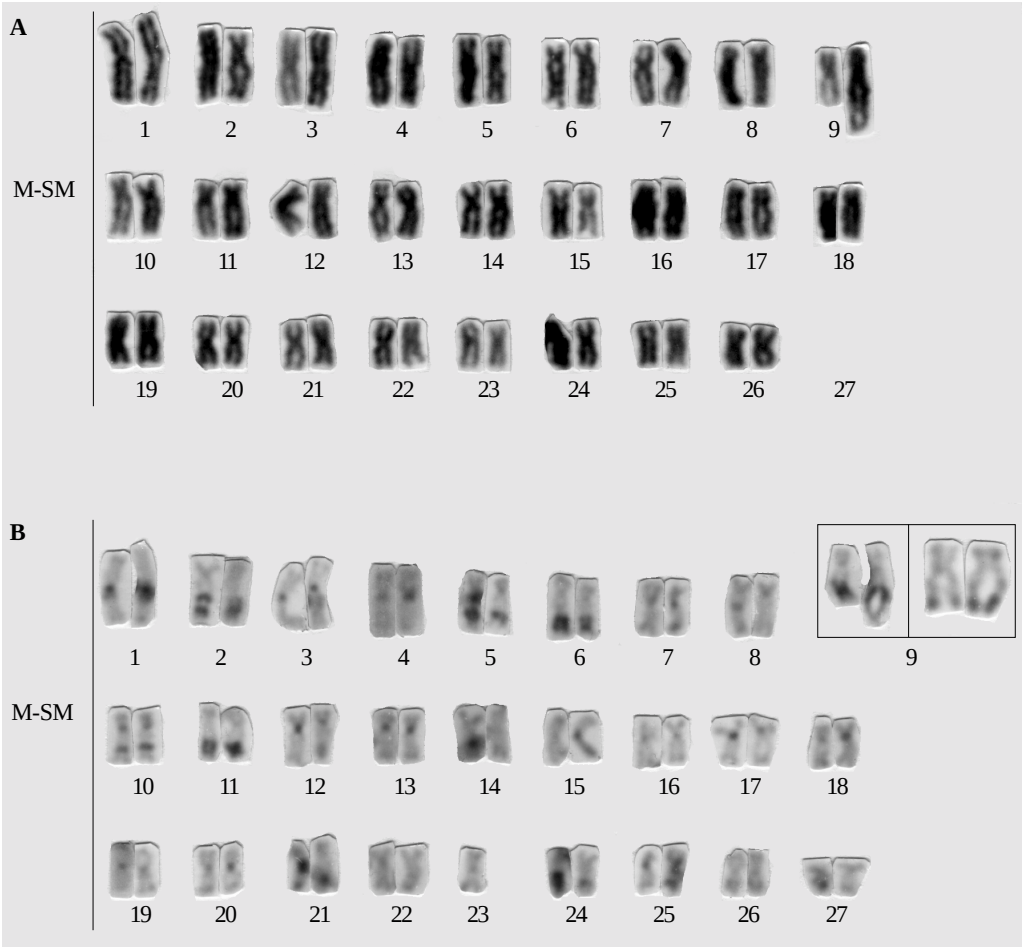

Figure 1 - Giemsa-stained (**A**) and C-banded (**B**) karyotype of *Leporinus desmotes* female. The NOR-bearing chromosomes (9th pair) are framed: C-banded (left) and silver nitrate stained (right).

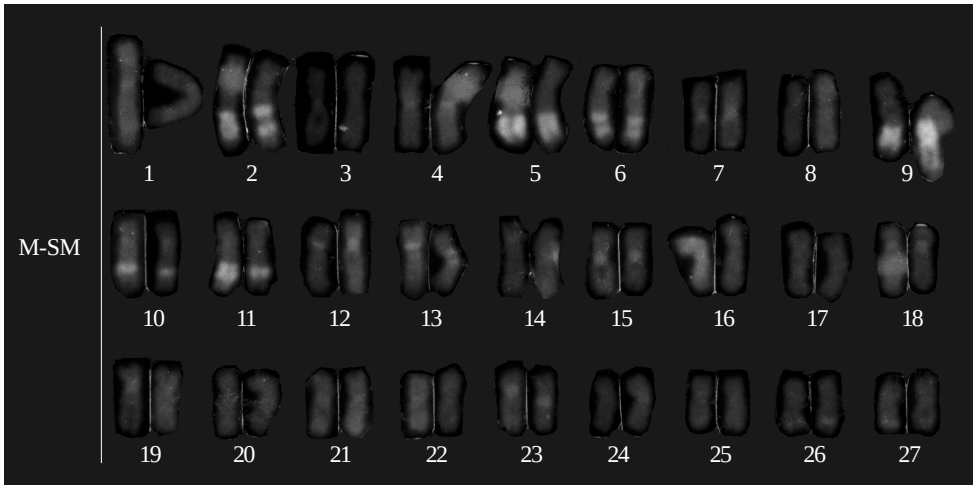

Figure 2 - Karyotype of *Leporinus desmotes* female stained with mithramycin.

band in this heterochromatin NOR-associated segment and in all of the intercalar heterochromatin segments (Figure 2). Conspicuous duplicated MM^+ segments were observed in the chromosomes 2 and 6, and were slightly apparent in the chromosome 5. A heteromorphism was detected in the pair 11, in which duplicated intercalar MM^+ bands were observed only in one element of the pair.

Fluorescent *in situ* hybridization (rDNA/FISH)

18S rDNA *in situ* hybridization showed fluorescent signals in only one chromosome pair (9th), confirming the presence of NORs in the end of the long arm of this chromosome pair (Figure 3). NOR size polymorphism was confirmed and no other minor NOR sites could be detected by FISH analysis.

DISCUSSION

The karyotype of *Leporinus desmotes* is very similar to the karyotype pattern previously described in other *Leporinus* species (Galetti *et al.*, 1981, 1984, 1991). However, a large amount of GC-rich heterochromatin was detected by MM staining among the chromosome complement of *L. desmotes.* This is an unusual occurrence in the chromosomes in the genus and more generally in fish. Often only NOR sites have been shown with MM^+ bands in the fish karyotypes (Schmid and Guttenbach, 1980; Mayr *et al.*, 1985; Phillips and Hartley, 1988; Sola *et al.*, 1992). In a few cases in other neotropical fish, some other minor fraction of the heterochromatin not associated with NOR showed MM^+ bands (Almeida-Toledo *et al.*, 1988; Artoni *et al.*, 1999).

The MM staining also revealed the NORs in the chromosome complement of *Leporinus desmotes*, confirmed by silver nitrate staining and 18S rDNA *in situ* hybridization. When a secondary constriction was present in the NOR area, stain intensity differences of both C- and MM banding were observed between the adjacent heterochromatin and the secondary constriction itself. The adjacent heterochromatin revealed C- and MM banding brighter than that of the secondary constriction. It was notable that the propidium iodide counterstaining used in FISH was able to show a differential color (approximately orange) of the NOR adjacent heterochromatin, similar to what has been previously reported to occur in other fish (Ràb *et al.*, 1996). The AgNOR-MM^+ relationship often observed in fish karyotypes has been explained by the presence of intertwined GC-rich heterochromatin along the rDNA arrays (Pendás *et al.*, 1993). However, the possibility of existence of NOR-associated heterochromatin may not be ruled out among fish (Artoni *et al.*, 1999).

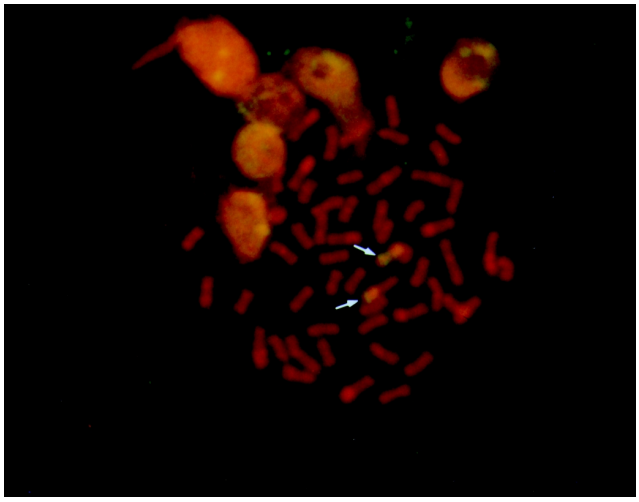

Figure 3 - Metaphase of *Leporinus desmotes* female observed after rDNA/FISH. The NOR-bearing chromosomes (9th pair) are indicated by arrows.

At least two categories of heterochromatin could be observed in the chromosomes of *L. desmotes*. First, there was the centromeric heterochromatin, which showed no fluorescence by MM staining. This is a common finding among fish (Schmid and Guttenbach, 1980; Mayr *et al.*, 1985; Phillips and Hartley, 1988; Galetti *et al.*, 1991; Sola *et al.*, 1992). The second category of heterochromatin was that seen joining the NOR adjacent-heterochromatin and the intercalar ones. This type was revealed by bright fluorescent MM-bands, indicating a typical GC-rich heterochromatin. This characterized the largest amount of heterochromatin observed in the genus *Leporinus*, outside of that related to sex chromosome differentiation. All *Leporinus* previously studied showed little heterochromatin, which was limited to the areas of the centromere and/or telomere (Galetti *et al.*, 1991). An exception is the case when the ZW sex chromosome system was detected (Galetti and Foresti, 1986; Galetti *et al*., 1995; Molina *et al*., 1998). Considering that no large differences in the euchromatic segments between related species are expected, the unique presence of this intercalar heterochromatin in *L. desmotes* suggests that these additional chromosome segments may have arisen by an extensive heterochromatin amplification, instead of resulting from chromatin substitution. In this way, it is possible that a primordial GC-rich heterochromatin, most probably the NOR-associated heterochromatin, could spread along some chromosomes by amplification processes. Potential mechanisms for this spread could be unequal chromosome exchanges, transpositions and regional duplications.

Further molecular studies might characterize satellite DNA occurring in this intercalar heterochromatin and eventually reveal a common origin of these chromosome segments in *L. desmotes*.

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RESUMO

Esta é a primeira descrição do cariótipo de *Leporinus desmotes* fêmea. O número diplóide encontrado foi 2n = 54 cromossomos meta- e submetacêntricos. As regiões organizadoras de nucléolos (NORs) foram estudadas através da impregnação pela prata e por hibridização *in situ* com sondas de DNAr (FISH), e foram localizadas na região telomérica do braço longo do 9º par. Heterocromatinas centromérica e telomérica foram reveladas pelo bandamento C na maioria dos cromossomos. Adicionalmente, grande quantidade de heterocromatina intercalar ou subtelomérica foi também observada. Diferenciação composicional na maior parte da heterocromatina identificada em *L. desmotes* pode ser inferida através da coloração pela mitramicina, caracterizando um caso peculiar de amplificação de segmentos heterocromáticos ricos em bases GC neste grupo de peixes.

REFERENCES

Almeida-Toledo, L.F., Foresti, F. and **Toledo-Filho, S.A.** (1988). An early stage of sex chromosome differentiation in the fish *Eigenmannia virescens* (Sternopygidae). *Proc. XVI Congrès Internat. Génét. Toronto, Canada (Abstract)* pp. 258.

Amemiya, C.T. and **Gold, J.R.** (1986). Chromomycin A_3 stains nucleolus organizer regions of fish chromosomes. *Copeia 1986*: 226-231.

Artoni, R.F., Molina, W.F., Bertollo, L.A.C. and **Galetti Jr., P.M.** (1999). Heterochromatin analysis in the fish species *Liposarcus anisitsi* (Siluriformes) and *Leporinus elongatus* (Characiformes). *Genet. Mol. Biol. 22*: 39-44.

Bertollo, L.A.C., Takashashi, C.S. and **Moreira Filho, O.** (1978). Cytotaxonomic considerations on *Hoplias lacerdae* (Pisces, Erythrinidae). *Rev. Bras. Genet. I*: 103-120.

Galetti Jr., P.M. and **Foresti, F.** (1986). Evolution of the ZZ/ZW system in *Leporinus* (Pisces, Anostomidae). Role of constitutive heterochromatin. *Cytogenet. Cell. Genet. 43*: 43-46.

Galetti Jr., P.M., Foresti, F., Bertollo, L.A.C. and **Moreira Filho, O.** (1981). Karyotypic similarity in three genera (*Leporinus, Leporellus, Schizodon*) of the family Anostomidae (Pisces, Teleostei). *Rev. Bras. Genet. IV*: 11-15.

Galetti Jr., P.M., Foresti, F., Bertollo, L.A.C. and **Moreira Filho, O.** (1984). Characterization of eight species of Anostomidae (Cypriniformes) fish on the basis of the nucleolar organizing region. *Caryologia 37*: 401-406.

Galetti Jr., P.M., Mestriner, C.A., Venere, P.C. and **Foresti, F.** (1991). Heterochromatin and karyotypic reorganization in fish of family Anostomidae (Characiformes). *Cytogenet. Cell. Genet. 56*: 116-121.

Galetti Jr., P.M., Mestriner, C.A., Monaco, P.J. and **Rash, E.M.** (1995). Postzygotic modifications and intra- and inter-individual nucleolar organizing region variations in fish: report of a case involving *Leporinus friderici*. *Chromosome Res. 3*: 285-290.

Hizume, M. (1994). Allodiplopid nature of *Allium wakegi* Araki revealed by genomic *in situ* hybridization and localization of 5S and 18S rDNAs. *Jpn. J. Genet. 69*: 407-415.

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.

Koehler, M.R., Dehm, D., Guttenbach, M., Nanda, I., Haaf, T., Molina, W., Galetti Jr., P.M. and **Schmid, M.** (1997). Cytogenetics of the genus *Leporinus* (Pisces, Anostomidae). 1. Karyotype analysis, heterochromatin distribution and sex chromosomes. *Chromosome Res. 5*: 12-22.

Margarido, V.P. and **Galetti Jr., P.M.** (1996). Chromosome studies in fish of the genus *Brycon* (Characiformes, Characidae, Bryconinae). *Cytobios 85*: 219-228.

Mayr, B., Kalat, M. and **Ràb, P.** (1985). Localization of NORs and counterstain-enhanced fluorescence studies in *Perca fluviatilis* (Pisces, Percidae). *Genetica 67*: 51-56.

Molina, W., Schmid, M. and **Galetti Jr., P.M.** (1998). Heterochromatin and sex chromosomes in the neotropical fish genus *Leporinus* (Characiformes, Anostomidae). *Cytobios 94*: 141-149.

Ojima, Y. and **Ueda, T.** (1979). New C-banded marker chromosomes found in carp-funa hybrids. *Proc. Jpn. Acad. Ser. B. 54*: 15-20.

Pendás, A.M., Moram, P. and **Garcia-Vazquez, E.** (1993). Ribosomal RNA genes are interspersed throughout a heterochromatic chromosome arm in Atlantic Salmon. *Cytogenet. Cell Genet. 63*: 128-130.

Phillips, R. and **Hartley, S.E.** (1988). Fluorescent banding patterns of the chromosomes of the genus *Salmo*. *Genome 30*: 193-197.

Pinkel, D., Straume, T. and **Gray, J.W.** (1986). Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. *Proc. Natl. Acad. Sci. USA 83*: 2934-2938.

Ràb, P., Reed, K.M., De Leon, F.A.P. and **Phillips, R.** (1996). A new method for detecting nucleolus organizer regions in fish chromosomes using

denaturation and propidium iodide staining. *Biotechnol. & Histochem. 71*: 157-163.

Sambrook, J., Fritsch, E.F. and **Maniats, T.** (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Schmid, M. (1980). Chromosome banding in Amphibia: IV. Differentiation of GC- and AT-rich chromosome regions in Anura. *Chromosoma 77*: 83-103.

Schmid, M. and **Guttenbach, M.** (1988). Evolutionary diversity of reverse (R) fluorescent chromosome bands in vertebrates. *Chromosoma 97*: 101-114.

Sola, L., Rossi, A.R., Iaselli, V., Rash, 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 mexicana* by C-banding and DAPI, quinacrine, chromomycin A_3 and silver staining. *Cytogenet. Cell. Genet. 60*: 229-235.

Souza, I.L., Moreira-Filho, O. and **Galetti Jr., P.M.** (1996). Heterochromatin differentiation in the characid fish *Astyanax scabripinnis*. *Braz. J. Genet. 19*: 405-410.

Sumner, A.T. (1972). A simple technique for demonstrating centromeric heterochromatin. *Exp. Cell Res. 75*: 304-306.

Sumner, A.T. (1990). *Chromosome Banding*. Unwin Hyman Ltd., London.

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