

Molecular cytogenetic study of heterochromatin in *Hisonotus leucofrenatus* (Teleostei, Loricariidae, Hypoptopomatinae)

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The fish species *Hisonotus leucofrenatus* exhibits a large amount of C-band positive segments with different responses after application of the C-banding technique. Type I class named herein appeared to be heavily stained after C-banding in the terminal position of five chromosome pairs and type II class, weakly stained after C-banding in the interstitial or pericentromeric position in nine chromosome pairs and in the supernumerary chromosomes. No variation was observed in type II C-band positive segments, however, type I segments displayed conspicuous polymorphisms, and six cytotypes were detected among the fish analyzed. Chromosomes were also analyzed by CMA₃ and DAPI staining, which showed that type I C-band positive segments comprised both AT-rich and GC-rich DNA, while type II segments were mainly composed of GC-rich sequences. *Hind*III-digested genomic DNA exhibits fragments of the ladder-like pattern, characteristic of tandemly arrayed repetitive sequences. Two of those fragments corresponding to monomeric and dimeric units of a 78 bp repetitive DNA sequence were cloned and sequenced. The cloned repetitive DNA was used as probe in fluorescent in situ hybridization experiments. The results revealed that these sequences were located in the same position as the type I C-band positive segments. This satellite DNA did not hybridize with DNA from other species of *Hisonotus* or from other fish of the family Loricariidae, suggesting that this sequence is specific to *H. leucofrenatus*. The role of these repetitive sequences in the karyotypic evolution of this species is discussed.

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Constitutive heterochromatin is composed of satellite DNA, a kind of in tandem organized repetitive DNA sequence, mainly as large clusters located in the centromeric and telomeric regions of the chromosomes (EPPLEN and EPPLEN-HAUPT 2002). These sequences have frequently been identified in chromosomes by the C-banding technique (SUMNER 1990). Satellite DNA represents non-coding DNA sequences organized as long arrays of head-to-tail linked repeats (PLOHL et al. 2008). Base-specific fluorochromes that stain AT or GC-rich DNA regions have been used to better understand the base composition of some C-band positive chromosome segments (MAISTRO et al. 1998b). The functional role of constitutive heterochromatin in the chromosome diversification process is not very well understood, however, several studies have shown that it may be related to chromosome polymorphisms (AMORES et al. 1993; MAISTRO et al. 1998b), different molecular compositions of heterochromatic segments (MAISTRO et al. 1998a), sex chromosomes differentiation (ANDREATA et al. 1992; MAISTRO et al. 1998a; PARISE-MALTEMPI et al. 2007), and the occurrence of supernumerary chromosomes (ANDREATA et al. 1993).

Satellite sequences have been isolated, cloned, marked with fluorescent compounds, and hybridized with

metaphasic chromosomes showing that its distribution is consistent with the distribution of C-band positive segments (REED and PHILLIPS 1995; DEVLIN et al. 1998; OLIVEIRA and WRIGHT 1998). This approach allows for the investigation of the origin, allocation, and evolutionary trends of these repetitive sequences in the karyotype (PHILLIPS and REED 1996). In Neotropical fishes, this kind of study has been carried out on freshwater species such as *Hoplias malabaricus* (HAAF et al. 1993) and saltwater species such as *Achirus lineatus* (AZEVEDO et al. 2005).

Cytogenetic studies in two populations of *Microlepidogaster leucofrenatus*, a species found in southeastern Brazilian coastal rivers and has now been allocated to the genus *Hisonotus*, evidenced the importance of C-band positive segments in the chromosome diversification and differentiation of this species (ANDREATA et al. 1993). Those two populations were differentiated by the amount and distribution of C-band positive segments, but both presented similar supernumerary chromosomes and a putative ZZ/ZW sex chromosome system identified by accumulation of the differential amount of C-band positive segments on the W chromosome. The objective of the present study was the analysis of DNA composition and distribution of C-band positive segments present

in autosomes, sex chromosomes, and supernumerary chromosomes of *H. leucofrenatus*, from the molecular and cytogenetic point of view.

MATERIAL AND METHODS

Cytogenetic studies were carried out on fish from two populations of *Hisonotus leucofrenatus*. The specimens were captured in coastal freshwater rivers of southeastern Brazil: three males and six females from the Marumbi River, Morretes, State of Paraná, and eight females from the Cavalo Stream, Jaraguá do Sul, State of Santa Catarina. The specimens were identified by Dr. Heraldo A. Britski (MZUSP) and deposited in the fish collection of the Laboratório de Biologia e Genética de Peixes, UNESP, Botucatu, São Paulo, Brazil.

Chromosome preparations and staining methods

Mitotic chromosome preparations were obtained from kidney and gill tissues using the air-drying technique (FORESTI et al. 1993). Nucleolus organizer regions (NORs) were identified by silver staining (Ag-NORs), as described by HOWELL and BLACK (1980). C-banding technique was accomplished according to SUMNER (1972); chromomycin A₃ and DAPI staining were done using metylgreen and distamicym A as counter-staining, respectively, following the protocols of SCHWEIZER (1976) and SCHWEIZER et al. (1978).

Satellite DNA isolation and cloning

Genomic DNA from a male individual of *Hisonotus leucofrenatus* from the Marumbi River was isolated and purified. Restriction enzyme digestions were conducted with the endonucleases *Hind*III, *Msp*I, *Pst*I, *Hae*III, *Eco*RI, *Pvu*II, *Sca*I, *Rsa*I and *Spe*I. The endonuclease *Hind*III generated a ladder of bands of approximately 80, 160, 240, 320 and 400 bp. Those DNA fragments were linked to the pUC18 and inserted into *Escherichia coli* DH5 α competent cells (Life technologies). Two recombinant clones, identified as Hleu1 and Hleu2, with inserts of about 80 and 160 bp were selected, sequenced, and used as probes in chromosome and membrane hybridization experiments.

DNA analysis

The two selected clones, Hleu1 and Hleu2, were sequenced with the Thermo Sequenase CY 5.5 Terminator Cycle Kit (GE Healthcare) using an Open Gene Automated DNA Sequencing System I (Visible Genetics) with the Gene Objects 3 software and deposited in the GenBank (accession no. GQ845170). For the Southern blotting analyses, genomic DNAs were partially and completely digested

with *Hind*III and transferred onto a nylon membrane according to Southern blotting method (SOUTHERN 1975). The membrane hybridization was conducted with the kit ECL-direct nucleic acid labeling and detection system (GE Healthcare) according to the manufacturer's recommendations.

Fluorescent in situ hybridization

FISH experiments were performed according to the method described by OLIVEIRA and WRIGHT (1998), with some modifications. DNA probe was labeled by nick translation with biotin-14-dATP, according to the manufacturer's instructions (Bionick™ Labeling System-Gibco. BRL). The satellite DNA sequences isolated in the present study and an 18S rDNA probe obtained from *Oreochromis niloticus* by one signatory of this paper (C. Oliveira) were located in the chromosomes with avidin-N-fluorescein isothiocyanate (FITC) conjugate (ONCOR), and the signal was enhanced using biotinylated anti-avidin goat antibodies following a second round of the

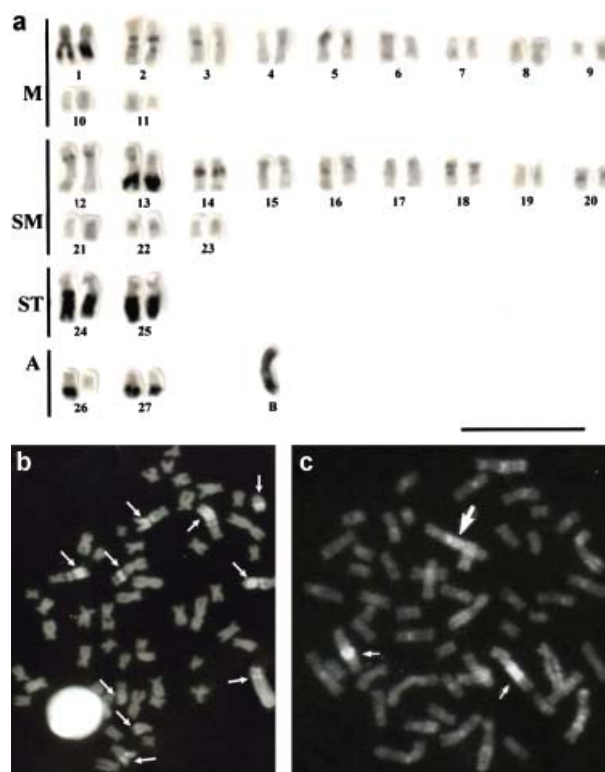


Fig. 1a–c. Chromosomes of *Hisonotus leucofrenatus*. (a) C-banded karyotype of a female ($2n = 55$) from the Marumbi River. The supernumerary chromosome is marked with letter “B”, bar = 10 μ m; (b) DAPI stained metaphase, the arrows indicate the bright stained terminal segments of chromosomes 13, 24, 25, 26 and 27; (c) CMA₃ (b) stained metaphase, the small arrows indicate NOR-bearing chromosomes, and the large arrow indicates the supernumerary chromosome.

Table 1. Possible different cytotypes of *Hisonotus leucofrenatus* that might be formed due to the polymorphisms observed in the chromosomes of pairs 25 and 26.

Cytotype	Heterochromatin distribution in the chromosomes		Occurrence
	25 / 25	26 / 26	
A	+ / +	+ / +	Marumbi River
B	+ / +	+ / -	Marumbi River
C	+ / +	- / -	Cavalo Stream
D	+ / -	+ / +	Not observed
E	+ / -	+ / -	Marumbi River
F	+ / -	- / -	Not observed
G	- / -	+ / +	Not observed
H	- / -	+ / -	Cavalo Stream
I	- / -	- / -	Cavalo Stream

(+) C-band positive segment present in the terminal region of the long arm of the chromosome. (-) C-band positive segment absent in the terminal region of the long arm of the chromosome.

avidin-FITC detection. Chromosomes were counterstained with propidium iodide. Metaphases were examined under a Zeiss Axiophot photomicroscope and pictures taken with a Kodak Gold Ultra 400 ASA film.

RESULTS AND DISCUSSION

The karyotypes of both populations of *Hisonotus leucofrenatus* studied evidenced the same karyotypic macrostructure, with $2n = 54$ chromosomes. One specimen from the Marumbi River presented a large additional chromosome characterized as supernumerary or B-chromosome (Fig. 1a). Such characteristics have already been observed and described in a previous study by ANDREATA et al. (1993, 2006).

The staining with C-banding technique showed that the chromosomes of *H. leucofrenatus* have at least two types of C-band positive segments: the first, type I, appeared heavily stained after C-banding (chromosome pairs 13, 24, 25, 26 and 27) and the second, type II, appeared weakly stained after C-banding (chromosome pairs 1, 2, 3, 5, 12, 16, 18, 20 and the supernumerary chromosome) (Fig. 1a). While no clear polymorphism had not been observed in the C-band positive segments of type II, evident polymorphisms were observed in the chromosome pairs 26 and 27 (Fig. 1a, 2). With regard to these polymorphisms, the occurrence of nine possible cytotypes (Table 1) was expected. Three of these cytotypes (A, B and E) (Table 1, Fig. 2) were found among the analyzed fish from the Marumbi River and three other cytotypes (C, H and I) (Table 1, Fig. 2, 7g) were found among the analyzed fish from the Cavalo Stream.

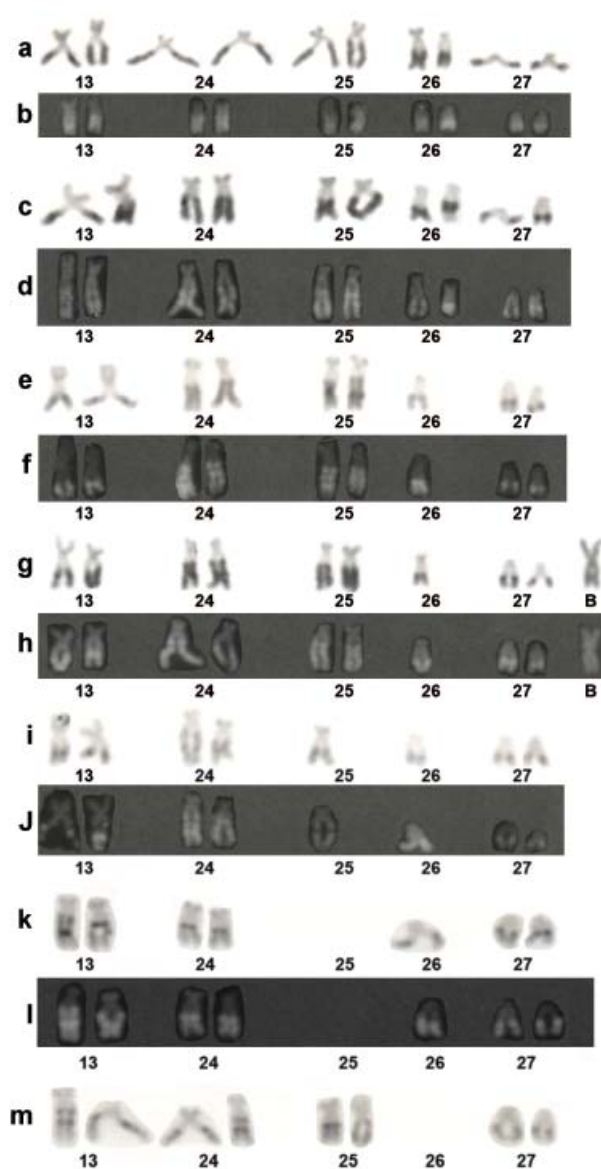


Fig. 2. C-banded and DAPI stained chromosomes of *Hisonotus leucofrenatus* from the Marumbi River (a-j) and the Cavalo Stream (l-m). (a) and (b) cytotype A - female; (c) and (d) cytotype A - male; (e) and (f) cytotype B - female; (g) and (h) cytotype B - female ($2n = 55$); (i) and (j) cytotype E - male; (l) C-banded and DAPI stained chromosomes (k) of cytotype H, (m) C-banded chromosomes of cytotype C.

Cytotypes B and C were the most frequent among the analyzed specimens in both the Marumbi River and the Cavalo Stream population, respectively (Table 2, 3). The high frequency occurring in cytotype B (56%) and its identification only in females of the Marumbi River population may be related to the identification of the ZZ/ZW sex chromosome system in this population (ANDREATA et al. 1993). This chromosome with a C-band positive

Table 2. Distribution and frequency of cytotypes of *Hisonotus leucofrenatus* from the Marumbi River.

Cytotype	No. of specimens males/females	Total	Frequency (%)
A	1/1	2	22
B	0/5	5	56
E	2/0	2	22

segment on the long arm was identified as the W chromosome in the study carried out by ANDREATA et al. (1993), in which only females were analyzed. However, in the present work, this sex-related system was not confirmed, and the existence of a heterochromatin polymorphic condition, on the other hand, is hypothesized.

Besides the evident polymorphism involving the terminal C-band positive segments of pairs 25 and 26, some minor polymorphisms were also observed in these chromosome pairs. Thus, in one specimen of the A cytotype from the Marumbi River, one of the two chromosomes of pair 26 presented an interstitial C-band positive segment, which is generally observed in this chromosome terminal position, suggesting the occurrence of a paracentric inversion (Fig. 2b).

The results of the staining of *H. leucofrenatus* chromosomes with DAPI were very similar to those obtained with the C-banding technique (Fig. 1b). Thence, at least two types of segments with different amounts of AT clusters are present in the genome of *H. leucofrenatus*: the first one, corresponding to the type I of C-band positive segments, was brightly stained after DAPI treatment (chromosome pairs 13, 24, 25, 26 and 27) and the second one, corresponding to the type II of C-band positive segments, was weakly stained after DAPI treatment (chromosome pairs 1, 2, 3, 5, 12, 13, 16, 18, 20 and the supernumerary chromosome) (Fig. 1c, 2). The staining with the fluorochrome CMA₃ displayed weak signals corresponding to all C-band positive segments (type I and II). Only the Ag-NOR segments and an interstitial segment in the supernumerary chromosome were brightly stained (Fig. 1c).

In the Marumbi River population, Ag-NORs were evidenced on only one pair of chromosomes, i.e. pair

number 1 (Fig. 3a–c). In specimens from the Cavalo Stream, Ag-NORs were also located on pair 1 (data not shown). Cytogenetic studies conducted with other species of *Hisonotus* and with other representatives of the subfamily Hypoptopomatinae revealed only one pair of chromosomes with interstitial Ag-NORs (ANDREATA et al. 1992, 1993, 1994, 2006; FERREIRA et al. 2005). One specimen from the Marumbi River showed a pericentric inversion involving one Ag-NOR-bearing chromosome (Fig. 3c). A similar characteristic was also observed in one fish from a sample of the species *Hisonotus* sp. A (ANDREATA et al. 2006).

The Ag-NORs of *H. leucofrenatus* were located close to a large heterochromatic segment in a euchromatic segment (Fig. 3a–c). Interestingly, the chromosome rearrangement observed in Fig. 3c did not involve the C-band positive segment, as observed in other fish species (FERNANDES-MATIOLI et al. 1997).

After staining of chromosomes with DAPI, the Ag-NOR-bearing chromosomes (pair 1) were similar to an unstained interstitial segment (a secondary constriction) not observed in the supernumerary chromosomes (Fig. 3d). After staining with CMA₃, the Ag-NOR-bearing chromosomes and the supernumerary chromosomes displayed a bright interstitial segment in a similar position (Fig. 1c). FISH experiments with the 18S rRNA gene probe showed that the Ag-NORs correspond to the location of the 18S rRNA gene on pair 1 and that the bright segment observed in the interstitial position of the supernumerary chromosomes does not present these genes (Fig. 3f).

The chromosome staining with GC-specific fluorochromes has been commonly associated with the identification of NORs in fish (MAYR et al. 1985; AMEMIYA and GOLD 1986), although in some cases the identification of heterochromatic GC-positive segments is not related to

Table 3. Distribution and frequency of cytotypes among females of *Hisonotus leucofrenatus* from the Cavalo Stream.

Cytotype	No. of specimens	Total	Frequency (%)
C	6	6	75
H	1	1	12.5
I	1	1	12.5

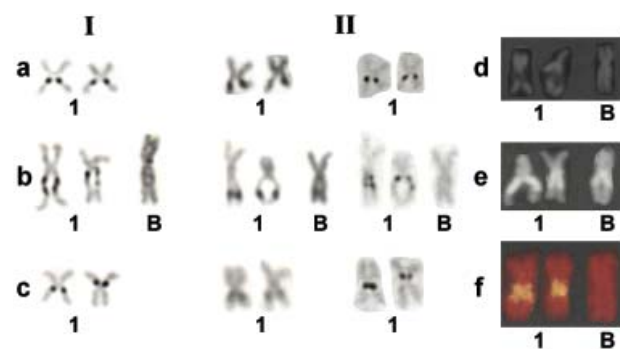


Fig. 3. NORs in chromosomes of *Hisonotus leucofrenatus* from The Marumbi River. (a) normal male; (b) female ($2n = 55$); (c) male with a pericentric inversion in the NOR-bearing chromosome. I - Ag-NORs; II - sequential staining C-band/Ag-NOR. (d–f) chromosomes of a female ($2n = 55$) stained with DAPI (d), CMA₃ (e) and FISH with 18S rRNA probe (f).

the occurrence of rDNA cistrons (ARTONI and BERTOLLO 1999). In *H. leucofrenatus*, this fluorochrome also stained pericentromeric regions of many chromosomes, which are DAPI negative (Fig. 1c). The supernumerary chromosome that is also DAPI negative, evidenced a large CMA₃ positive block similar to that observed in the NOR bearing chromosome. This fact suggests a possible correspondence between those chromosomes and that the GC-rich region of the supernumerary chromosome does not present 18S rRNA genes (Fig. 3f).

Restriction endonuclease digestions were performed on the genome of *H. leucofrenatus* to isolate repetitive sequences and comparisons of them with the C-band localization of the studied species were conducted. When the genomic DNA of *H. leucofrenatus* was digested with *Hind*III, some bands (from about 80 bp to 400 bp) were observed in agarose gels (data not shown). The cloning of two of these bands revealed the existence of two repetitive DNA sequences, named Hleu1 (78 bp) and Hleu2 (158 bp), constituted by a monomer and a dimer, respectively (Fig. 4a–b). The comparative analysis of the sequences obtained displayed very a high homology among them, with a predominance of AT-base pairs (61%) (Fig. 4b). The Southern blotting experiment with DNA of *H. leucofrenatus* digested with *Hind*III during different times (Fig. 5a) showed that these sequences are distributed in tandem arrays, characteristic of a satellite DNA family (Fig. 5b). The analysis of DNA from other Loricariidae species, including a different species of *Hisonotus*, showed that the

sequence found in *H. leucofrenatus* is not present in any of the four species tested (Fig. 5b).

The FISH experiments with Hleu2 probe showed that this satellite DNA was only located on the long arm of chromosome pairs 13, 24, 25, 26 and 27 (Fig. 6, 7), corresponding to the type I C-band positive segments. The polymorphisms evidenced with the C-banding technique were also observed in FISH experiments (Fig. 7).

The distribution of Hleu2 sequences in the terminal position of several chromosome pairs, as observed in *H. leucofrenatus*, is uncommon in fish, since many studies have related the existence of satellite sequences located in pericentromeric regions of the chromosomes (REED and PHILLIPS 1995; OLIVEIRA and WRIGHT 1998; JESUS et al. 2003). On the other hand, a study conducted with *Parodon hilari* showed that a 200 bp satellite sequence was located in the terminal C-band positive blocks of four autosomic pairs, in the terminal position of the long arm of the Z chromosomes, and in the terminal position of the short arm of the W chromosomes (VICENTE et al. 2003).

The analysis of the type I C-band positive segments of *H. leucofrenatus* showed that this kind of chromatin was brightly stained with DAPI (Fig. 1b), as expected, since the Hleu2 satellite sequence was 61% AT-rich, but the same kind of chromatin also exhibited some affinity to the fluorochrome CMA₃ (Fig. 1c). These facts suggest that in the type I C-band positive chromatin some AT-rich (Hleu2 sequence) and some GC-rich sequences are interspersed.

a

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Hleu2  TGTAAGTATAACTAAGGCTAGGAGTCGACTCTCCGAATCTCCGATTCACTGTTATATCAA 60
Hleu1  TGTAAGTATTACTAAGGCTAGGAGTCGACTCTCCGAATCTCTGATTCACTGTTATATCAA 60
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*****

Hleu2  TTGATTCATTTCTTAAGCTTTTAAGTATAACTAAGGCTAGGAGTCGACTCTTCGAATCTC 120
Hleu1  TTGATTCATTTCT-AAGCT----- 78
*****

Hleu2  CGATTCACTTTTATATCAATTGATTCATTTTTTAAGCT 158
Hleu1  -----

b

Hleu1  TGTAAGTATTACTAAGGCTAGGAGTCGACTCTCCGAATCTCTGATTCACTGTTATATCAA 60
Hleu2-A TGTAAGTATAACTAAGGCTAGGAGTCGACTCTCCGAATCTCCGATTCACTGTTATATCAA 60
Hleu2-B TTTAAGTATAACTAAGGCTAGGAGTCGACTCTTCGAATCTCCGATTCACTTTTATATCAA 60
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Hleu1  TTGATTCATTTCT-AAGCT 78
Hleu2-A TTGATTCATTTCTTAAGCT 79
Hleu2-B TTGATTCATTTTTTAAGCT 79
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Fig. 4. DNA sequences of the clones Hleu1 (78 pb) and Hleu2 (158 bp) of *Hisonotus leucofrenatus*. (a) Alignment of Hleu1 and Hleu2 sequences. (b) comparative analysis between the sequence of Hleu1 and the sequences of monomers A and B of Hleu2. The underlined regions correspond to the cleavage site of *Hind*III endonuclease.

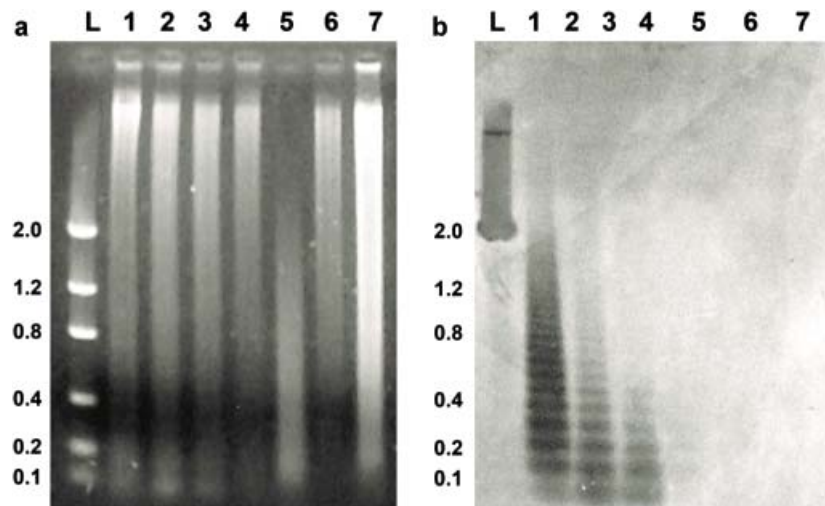


Fig. 5a–b. (a) Agarose gel displaying the DNA of *Hisonotus leucofrenatus* from the Marumbi River digested with *Hind*III during 10 min. (1), 60 min. (2), and overnight (3). In lines 4, 5, 6 and 7, DNA of *Hisonotus* sp. A from the Claro River, *Neoplecostomus paranaensis*, *Rinelepis aspera*, and *Hypostomus* sp. digested overnight with *Hind*III. (b) Southern blot experiment showing the DNA of agarose gel hybridized with the Hleu2 probe. L = molecular weight marker (kb).

The signal observed in the FISH experiments with Hleu2 probe was heterogeneous reinforcing the hypothesis of the existence of different sequences interspersed in these chromosome regions (Fig. 6, 7).

Numerical and size polymorphisms of the heterochromatin, such as those observed in *H. leucofrenatus*, can be explained through some evolutionary models that postulate the increase in heterochromatic regions and its distribution among the chromosomes. In the dispersion of

the heterochromatin model proposed by SCHWEIZER and LOIDL (1987), the proximity of the chromosomes during mitotic anaphases could allow for the transfer of heterochromatic segments between chromosome regions that are equidistant from centromeres. This model seems to apply to *H. leucofrenatus* populations, because polymorphic blocks of heterochromatin are found at the end of several chromosomes in this species and their distribution appear to be governed by concerted evolution mechanisms. However, the occurrence of other models, such as the unequal chromatid exchange and amplification and deletion events of the heterochromatic regions, cannot be discarded. The dispersion of the heterochromatic model proposed by SCHWEIZER and LOIDL (1987) was applied to explain the C-band polymorphisms observed in the karyotypes of two populations of *Astyanax scabripinnis* (MANTOVANI et al. 2000) and in *Physalaemus petersi*, in which this model is related to the polymorphic NORs found in heterochromatic regions of the chromosomes (LOURENÇO et al. 1998).

The data obtained in the present study evidenced that constitutive heterochromatin of *H. leucofrenatus* comprise at least two distinct components: one that is AT-rich and other that is GC-rich. The AT-rich satellite sequences of *H. leucofrenatus* are interesting because they were not found in any other related loricariids studied, and conspicuous differences including several polymorphisms were observed among fish from the two studied samples, suggesting that changes in the distribution of these satellite sequences are very dynamic and may be related to the chromosome differentiation observed in this species.

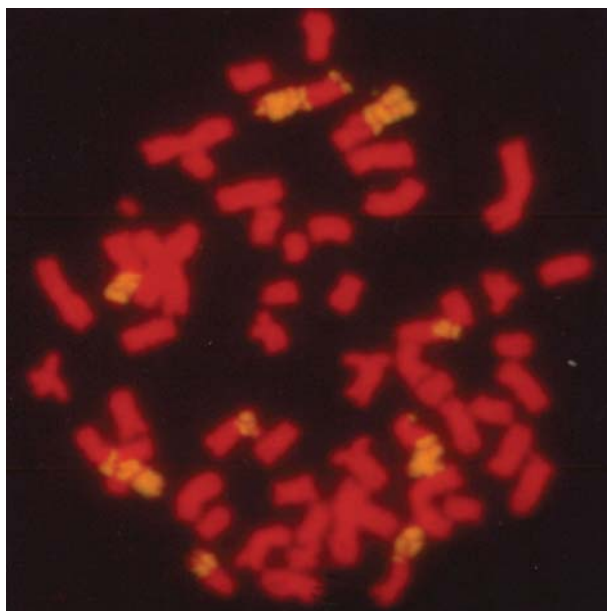


Fig. 6. Metaphase of *Hisonotus leucofrenatus* from the Marumbi River hybridized with the repetitive Hleu2 probe.

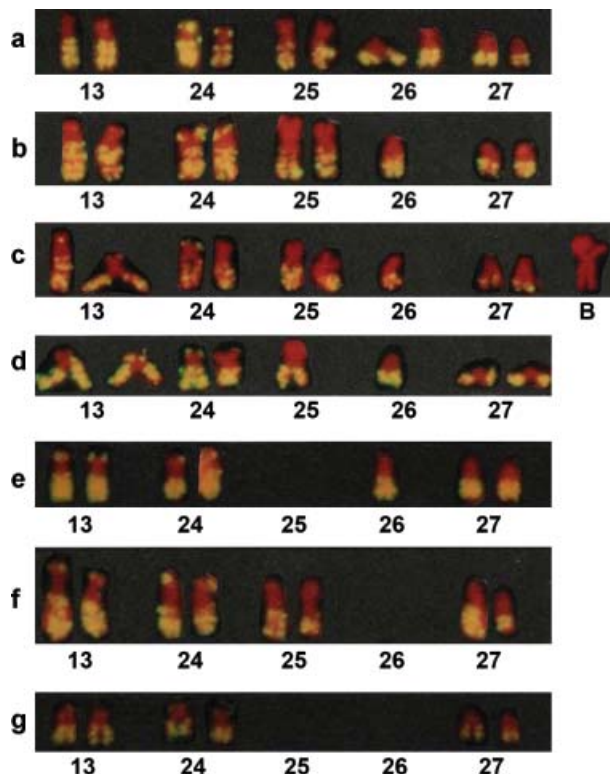


Fig. 7. Chromosomes of *Hisonotus leucofrenatus* from the Marumbi River (a–d) and the Cavalo Stream (e–g) hybridized with Hleu2 probe. (a) cytotype A – female, (b) cytotype B – female, (c) cytotype B – female ($2n = 55$), and (d) cytotype E – male, (e) cytotype H, (f) cytotype C and (f) cytotype I.

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