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ORIGINAL PAPER

Chromosomal mapping of repetitive sequences in *Hyphessobrycon eques* (Characiformes, Characidae): a special case of the spreading of 5S rDNA clusters in a genome

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Abstract

Cytogenetic data showed a variation in diploid chromosome number in the genus *Hyphessobrycon* ranging from 2n = 46 to 52, and studies involving repetitive DNA sequences are scarce in representatives of this genus. The purpose of this paper was the chromosomal mapping of repetitive sequences (rDNA, histone genes, U snDNA and microsatellites) and investigation of the amplification of 5S rDNA clusters in the *Hyphessobrycon eques* genome. Two *H. eques* populations displayed 2n = 52 chromosomes, with the acrocentric pair No. 24 bearing Ag-NORs corresponding with CMA_3+/DAPI−. FISH with a 18S rDNA probe identified the NORs on the short (*p*) arms of the acrocentric pairs Nos. 22 and 24. The 5S rDNA probe visualized signals on almost all chromosomes in genomes of individuals from both populations (40 signals); FISH with H3 histone probe identified two chromosome pairs, with the pericentromeric location of signals; FISH with a U2 snDNA probe identified one chromosome pair bearing signals, on the interstitial chromosomal region. The mononucleotide (A), dinucleotide (CA) and tetranucleotide (GATA) repeats were observed on the centromeric/pericentromeric and/or terminal positions of all chromosomes, while the trinucleotide (CAG) repeat showed signals on few chromosomes. Molecular analysis of 5S rDNA and non-transcribed spacers (NTS) showed microsatellites (GATA and A repeats) and a fragment of retrotransposon (SINE3/*5S-Sauria*) inside the sequences. This study expanded the available cytogenetic data for *H. eques* and demonstrated to the dispersion of the 5S rDNA sequences on almost all chromosomes.

Keywords U2 snDNA · Microsatellites · 18S rDNA · H3 histone · *5S-Sauria*

Introduction

Hyphessobrycon represents one of the largest genus in the family Characidae and it comprises 143 recognized species (Froese and Pauly 2019). Native to the Neotropical region, the species of the genus *Hyphessobrycon* are widely distributed from southern Mexico to Argentina (Rio de la Plata) (Lima et al. 2003). Data based on morphological and molecular characteristics of Characidae indicate that *Hyphessobrycon* does not form a monophyletic group (Mirande 2010; Javonillo et al. 2010; Oliveira et al. 2011) and presently, it is placed in the subfamily Stethaprioninae (Eschmeyer and Fong 2019). Approximately 30% of the *Hyphessobrycon* species are of commercial interest because they exhibit an attractive coloration pattern (Castro-Paz et al. 2014).

The genus *Hyphessobrycon* includes a large number of recognized species, but few of them have been

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cytogenetically studied. In spite of this fact, they have shown highly diverse karyotypes in terms of different both diploid number (2n) and karyotype structures. Among the studied representatives of this genus, the diploid number ranges from 2n = 46 in *H. tropis* (Sheel 1973) to 2n = 52 in *H. eques* (Martinez et al. 2012; Piscor and Parise-Maltempi 2015). Moreover, B chromosomes of the size of microchromosomes were revealed in genomes of *H. eques* individuals collected from the Paraná River basin (Piscor and Parise-Maltempi 2015).

The studies involving repetitive DNA sequences in the genus *Hyphessobrycon* are scarce, with data available only for 18S rDNA in *H. anisitsi* (Centofante et al. 2003; Mendes et al. 2011) and *H. luetkenii* (Mendes et al. 2011); and for 5S rDNA only in a single species, *H. anisitsi* (Centofante et al. 2003). Other repetitive DNA sequences such as histone genes, genes coding for small nuclear RNAs (U snDNA) and microsatellites have not been investigated in genomes of the species of this genus.

Considering that the patterns of repetitive DNA distribution are important for the study of chromosomal evolution, in fishes, this information has contributed substantially to the understanding of trends and dynamics of chromosomal evolution (Mestriner et al. 2000; Cioffi and Bertollo 2010; Symonová et al. 2013; Poltronieri et al. 2014; Sember et al. 2015; Yano et al. 2016; Fernandes et al. 2017; Glugoski et al. 2018; Soto et al. 2018). The purpose of this paper was the analyses of chromosomal locations of repetitive sequences (rDNA, histone genes, U snDNA and microsatellites) and investigation of the amplification of 5S rDNA clusters in the genome of *H. eques*.

Materials and methods

Samples and statement of ethics

Individuals from two populations of *H. eques* were collected for cytogenetic and molecular analyses: six males and four females from the Piracicaba River (Santa Maria da Serra—SP); seven males and four females from the Ribeirão Claro River (Rio Claro—SP).

All the institutional guidelines for the care and use of laboratory animals were followed. The animals were captured with permission from the Instituto Chico Mendes de Conservação da Biodiversidade—ICMBio (process number—43497-1) and used for laboratory experiments approved by the Animal Experimental Ethics Committee from the Universidade Estadual Paulista 'Júlio de Mesquita Filho'—UNESP (protocol number—2335).

Conventional chromosome analyses

The chromosomes were prepared according to Foresti et al. (1981). The morphology of the chromosomes was determined according to the arm's ratio (Levan et al. 1964). The fundamental number (FN) was calculated according to the chromosomal arm numbers (the m, sm and st chromosomes were considered biarmed—*p* and *q* arms—and a chromosomes were considered uniarmed—only *q* arm. Note that a correspond to acrocentric). The nucleolar organizer regions (NORs) were detected using the silver nitrate impregnation technique described by Howell and Black (1980). Heterochromatin was observed using the C-banding technique of Sumner (1972). CG- and AT-rich regions were identified by double-color Chromomycin A_3/4',6-diamidino-2-phenylindole (CMA_3/DAPI) staining with denatured chromosomes, according to a technique commonly used in the Cytogenetic Laboratory of the Universidade Estadual Paulista 'Júlio de Mesquita Filho' (UNESP) in Rio Claro/SP (for more detail see, Piscor et al. 2015).

DNA extraction and production of probes

The genomic DNAs (gDNA) were extracted from fin samples of *Astyanax* and *Hyphessobrycon* individuals, as described by Sambrook and Russell (2001). The 18S rDNA, 5S rDNA, H3 histone and U2 snDNA probes were obtained by PCR using primers, described by White et al. (1990), Pendás et al. (1994), Cabral-de-Mello et al. (2010) and Bueno et al. (2013), respectively. Moreover, considering the high coincidence of many repetitive sequences, we have tested also FISH with 5S rDNA from other fish species (e.g. *Astyanax altiparanae*, *A. fasciatus*, *Piabina argentea* and *Megaleporinus elongatus*) in order to discard the possibility that our probe prepared from gDNA of *H. eques* was insufficient for the experiment due to diverse repetitive DNA content. The probes were labeled by PCR with digoxigenin-11-dUTP (Roche Applied Science, Penzberg, Germany) or biotin-16-dUTP (Roche Applied Science).

The $(A)_{30}$, $(CA)_{15}$, $(CAG)_{10}$ and $(GATA)_8$ microsatellites were amplified and labelled with biotin during synthesis as described by Milani and Cabral-de-Mello (2014). Microsatellites were donated by Prof. Dr. Diogo C. Cabral-de-Mello .

Technique of fluorescent in situ hybridization (FISH)

The FISH technique followed Pinkel et al. (1986), with modifications described by Margarido and Moreira-Filho (2008). Signals were detected using anti-digoxigenin–Rhodamine (Roche Applied Science) for digoxigenin-11-dUTP and avidin–FITC (Sigma Aldrich, St Louis, MO, USA) for

biotin-16-dUTP (Roche Applied Science). The chromosomes were counterstained with DAPI. Metaphases were photographed using a BX 61 epifluorescence microscope, coupled with an Olympus DP 71 digital camera (Olympus America, Inc.) with Olympus DP Controller software 3.2.1.276.

Cloning, sequencing and analysis of 5S rDNA-NTS sequences

The PCR products of 5S rDNA amplification were cloned using competent bacteria according to chemical transformation with $CaCl_2$. The DNA fragments were inserted into the plasmid vector with pGEM®-T kit (Promega, Madison, WI, USA) following the manufacturer's specifications. The clones were purified by treatment with ExoSAP-IT® (USB) and sent to sequencing service using the same primers of the PCR reaction as the international platform for sequencing, Macrogen Company (South Korea).

The sequences were edited and analyzed using the BioEdit (Hall 1999) program and the Clustal W algorithm (Thompson et al. 1994) for performing alignment of the sequences. For the identification of the sequences, the CENSOR tool (https://www.girinst.org) (Kohany et al. 2006) and nucleotide BLAST tool (NCBI - National Centre for Biotechnology Information) were used. Finally, the sequences were deposited in GenBank (access numbers: MN396769 to MN396772).

Results

The individuals of *H. eques* from the Piracicaba River 2n = 52 and karyotype composed of 10 m + 20sm + 8st + 14a chromosomes, with NF = 90 (Fig. 1a). Ag-NORs and CMA_3 positive sites were located on the *p* arm of pair No. 24 (Fig. 1a, in box). The 18S rDNA clusters were located on the *p* arms of pairs Nos. 22 and 24 (Fig. 1b, in box). The heterochromatin regions were observed in the centromeric positions of almost all chromosomes (Fig. 1b).

The 5S rDNA clusters were observed on almost all chromosomes (20 pairs) in pericentromeric positions in the individuals from both populations (Fig. 2). Four sites of H3 histone were observed on the pericentromeric regions in two pairs, where in one pair the histone H3 cluster was in a position adjacent to 5S rDNA (Fig. 3a). The U2 snDNA clusters were identified on the interstitial positions of the *q* arm of one chromosome pair, also adjacent to 5S rDNA (Fig. 3b). Microsatellites $(A)_{30}$ and $(GATA)_8$ were detected mainly in the centromeric regions of the chromosomes (Fig. 3c, f). The microsatellite $(CAG)_{10}$ showed some signals on the short/long arms of chromosomes but no scatter pattern (Fig. 3d),

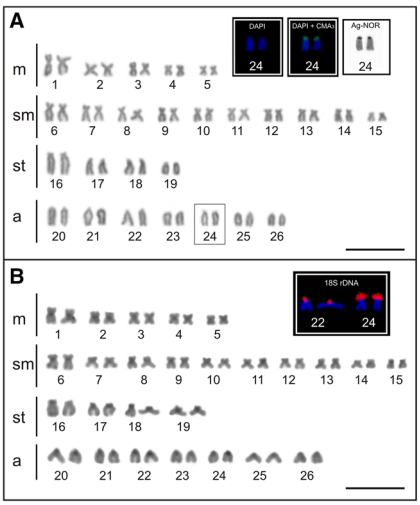

Fig. 1 Karyotypes of the *Hyphessobrycon eques* from the Piracicaba River arranged from: **a** Giemsa stained; **b** C-banded chromosomes. Note that DAPI-negative, CMA_3-positive, Ag-NOR chromosomes and 18S rDNA sites are indicated in the boxes. Bar = 10 µm

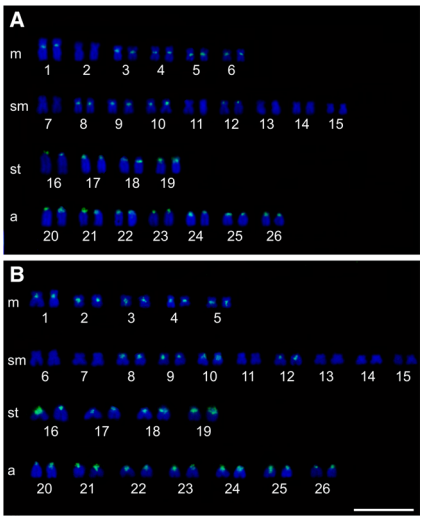

Fig. 2 **a** Karyotype of *Hyphessobrycon eques* from the Ribeirão Claro River (Rio Claro—SP); **b** Karyotype of *Hyphessobrycon eques* from the Piracicaba River (Santa Maria da Serra—SP) arranged from chromosomes with fluorescent signals of the 5S rDNA probe. Bar = 10 µm

with $(CA)_{15}$ mainly on the terminal regions of chromosomes (Fig. 3e).

The sequence clones of NTS regions (D1, D2, D3 and D4) showed around 300 bp (Fig. 4). Inside the sequences we identified fragments with 97 bp and approximately 97% similarity for the retrotransposon SINE3/*5S-Sauria*, SINE (Short Interspersed Nuclear Element), Non-LTR (Non-Long Terminal Repeat) and microsatellite repeats, e.g. GATA and A repeats (Fig. 4).

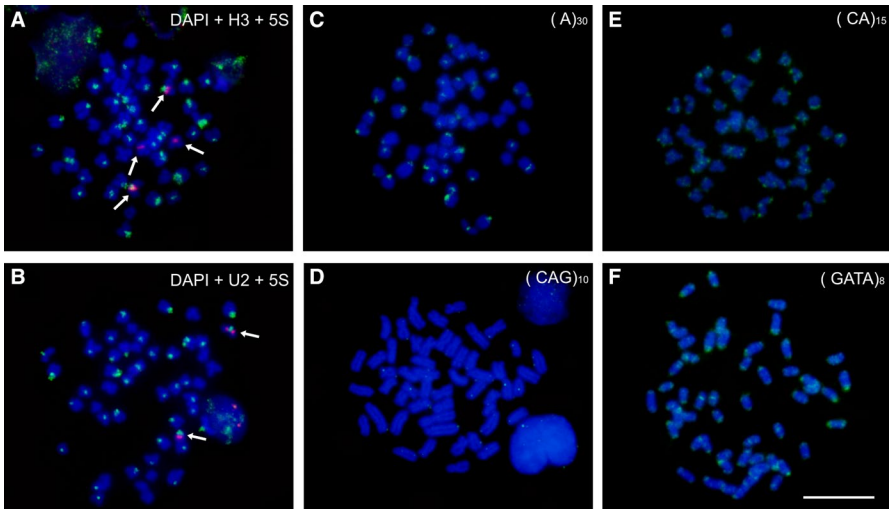

Fig. 3 Repetitive DNA sequences in genome of *Hyphessobrycon eques* from the Piracicaba River (Santa Maria da Serra—SP). **a** Metaphase with 5S rDNA clusters in green and histone H3 in red. **b** Metaphase with 5S rDNA clusters in green and U2 snDNA in red. **c** Microsatellite $(A)_{30}$ in green. **d** Microsatellite $(CAG)_{10}$ in green. **e** Microsatellite $(CA)_{15}$ in green. **f** Microsatellite $(GATA)_8$ in green. Bar = 10 µm

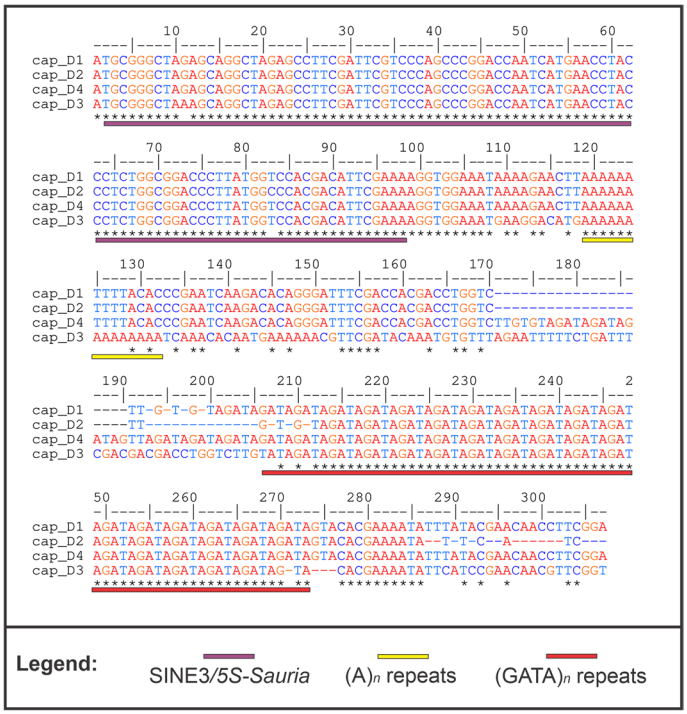

Fig. 4 Clones of the 5S rDNA-NTS sequences of two *Hyphessobrycon eques* populations. D1 and D2 from the Ribeirão Claro River (Rio Claro—SP) and D3 and D4 from the Piracicaba River (Santa Maria da Serra—SP). Note that the A repeats are located inside the D3 sequence only.

Discussion

Our results showed that individuals from populations of *H. eques* from the two analyzed locations had the same 2n = 52 and FN = 90. Although having the same 2n = 52, the individuals of *H. eques* from the Capivara River reported previously (Martinez et al. 2012) differed in their FN values. For example, the number of a chromosomes—14 elements (FN = 90) in the populations under study and 18 elements (FN = 86) from the Capivara River (Martinez et al. 2012). This may perhaps be due to different classification of the chromosome categories

Heterochromatin has been observed mainly on the centromeric/pericentromeric regions of almost all chromosomes of *H. eques* from the Ribeirão Claro River (Piscor and Parise-Maltempi 2015). In this paper, similar patterns of constitutive heterochromatin were identified on the chromosomes of *H. eques* from the Piracicaba River. Another species of the genus, *Hyphessobrycon reticulatus* (Carvalho et al. 2002), *H. anisitsi* and *H. luetkenii* (Mendes et al. 2011), also possessed karyotypes with constitutive heterochromatin in the pericentromeric regions of all or almost all chromosomes, indicating that this pattern may be a characteristic feature of their genomes.

Ag-NOR sites corresponded to CMA_3^+ signals on the *p* arms in the terminal position of the a chromosome pair No. 24 in individuals under this study. Similarly, the individuals from population from the Capivara River (Martinez et al. 2012) also had karyotypes one Ag-NOR site corresponding to CMA_3^+ in terminal position on the *p* arm of the pair No. 17, demonstrating that the NOR regions in *H. eques* were interspersed with GC-rich sequences, as well as in genomes of other fish species (Mayr et al. 1985; Amemiya and Gold 1986; Galetti et al. 1995; Fernandes and Martins-Santos 2004; Fernandes et al. 2015).

Our FISH results, represented the first physical mapping of repetitive sequences (rDNA, histone genes, U snDNA and microsatellites) in genome of *H. eques*. H3 histone clusters were observed on two chromosome pairs, one pair holds syntenic sequences of H3 histone and 5S rDNA. Synteny of H3 histone and 5S rDNA clusters was also described in genome of *Astyanax* (Piscor and Parise-Maltempi 2016). The *H. eques* also had a syntenic location of U2 snDNA and 5S rDNA in one pair of chromosomes. On the other hand, the chromosomal locations of the 5S rDNA and U2 snDNA clusters in genome of *Astyanax* were not consistently linked (Piscor et al. 2016). This spatial separation of 5S rDNA and U2 snDNA clusters appears to be the most frequent pattern in fish chromosomes (Merlo et al. 2012; Yano et al. 2017; Sember et al. 2018; Piscor et al. 2018). However, a synteny of these repetitive sequences in genome of *H. eques* could be related to the high number of 5S rDNA clusters .

The 18S rDNA clusters were observed in four chromosomes of karyotype of *H. eques* from the Piracicaba River, while in that of *H. anisitsi*, 18S rDNA signals were observed in ten chromosomes (Centofante et al. 2003). The transposable elements can move *via* transposition and/or ectopic recombination taking of rDNA sequences to other sites (Raskina et al. 2004, 2008). Similarly, we demonstrated an extensive dispersion of 5S rDNA sequences in individuals from two *H. eques* populations, which might also be influenced by the activity of transposable elements.

Physical mapping of 5S rDNA in the genome of *H. eques* showed these clusters distributed on 40 chromosomes in individuals from both analyzed populations, while in formerly investigated *H. anisitsi*, 5S rDNA were observed in four chromosomes (Centofante et al. 2003). Other studies showed the 5S rDNA clusters on several chromosome pairs of another fish species (see, for example, Cioffi et al. 2010; Nakajima et al. 2012; Sember et al. 2015; Silva et al. 2016). In *Gymnotus mamiraua* (Gymnotiformes, Gymnotidae) with 2n = 54, more than half of them had syntenic localisation with *Tc1/Mariner* transposon and 5S rDNA signals, possibly indicating a pseudogene (Silva et al. 2016). In other examples, the spreading of 5S rDNA clusters have led subsequently to development of specific centromeric satellite DNA in genome of *Hoplias malabaricus* (Erythrinidae) (Martins et al. 2006), while highly amplified 5S rDNA loci in two sister species of *Esox* (Esociformes, Esocidae) seem to retained functionality, as their sequence is not degenerated into pseudogenes; and the expression of additional copies seems to be regulated by DNA methylation (Symonová et al. 2017).

The NTS regions are subject to intense modification and rapid evolution, resulting in deletions, insertions and substitutions, as well as the inclusion of pseudogenes and microsatellites (Eickbush and Eickbush 2007; Pinhal et al. 2011; Rebordinos et al. 2013; Silva et al. 2016). In this paper, microsatellite repeats were also shown in NTS regions in two populations de *H. eques* (Ribeirão Claro River and Piracicaba River), as well as fragments of transposable element (Non-LTR, SINE3/*5S-Sauria*), probably responsible for spreading 5S rDNA sequences on almost all chromosomes. Merlo et al. (2013) showed that different NTS types may contain pseudogenes, LTR-*Gypsy*, non-LTR (LINE—Long Interspersed Nuclear Element) and microsatellites in *Diplodus sargus* (Sparidae). Thus, the latter authors suggest that the concerted evolution model does not explain the 5S rDNA variability of *D. sargus*; however, the birth-and-death evolution model could explain it.

Mechanisms such as concerted and birth-and-death models have been proposed for explaining the evolution of multigene families (Nei and Rooney 2005). Similarly, Pinhal et al. (2011) pointed out that 5S rDNA molecular evolution in fish genomes is driven by a mixed mechanism that integrates birth-and-death and concerted evolution models. Both these mechanisms could explain the evolution of 5S rRNA genes in genome of *H. eques*.

Our data of the physical mapping of repetitive sequences in the genome of *H. eques* genome showed one pair that holds syntenic sequences of H3 histone and 5S rDNA and the other pair holds syntenic sequences of U2 snDNA and 5S rDNA. The 18S rDNA clusters were observed on four chromosomes, while we demonstrated an extensive dispersion of 5S rDNA sequences, with microsatellite sequences and transposable elements identified in the NTS regions. Thus, the dispersion of 5S rDNA clusters on almost all chromosomes was an indication that this form of organization may be favorable for the ongoing elevated genome dynamic in *H. eques* .

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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