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Repetitive DNA in the catfish genome: rDNA, microsatellites, and tc1-mariner transposon sequences in *Imparfinis* species (Siluriformes, Heptapteridae)

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Short running title: Repetitive DNA in the catfish genome

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Abstract

Physical mapping of repetitive DNA families in the karyotypes of fish is important to understand the organization and evolution of different orders, families, genera, or species. Fish in the genus *Imparfinis* show diverse karyotypes with various diploid numbers and ribosomal DNA (rDNA) locations. Here we isolated and characterized Tc1-mariner nucleotide sequences from *Imparfinis schubarti*, and mapped their locations together with 18S rDNA, 5S rDNA, and microsatellite probes in *Imparfinis borodini* and *I. schubarti* chromosomes. The physical mapping of Tc1/Mariner on chromosomes revealed dispersed signals in heterochromatin blocks with small accumulations in the terminal and interstitial regions of *I. borodini* and *I. schubarti*. Tc1/Mariner was coincident with rDNA chromosomes sites in both species, suggesting that this transposable element may have participated in the dispersion and evolution of these sequences in the fish genome. Our analysis suggests that different transposons and microsatellites have accumulated in the *I. borodini* and *I. schubarti* genomes and that the distribution patterns of these elements may be related to karyotype evolution within *Imparfinis*.

Keywords: MITEs, transposable elements, karyotype evolution, genome evolution.
Subject area: Genomics and gene mapping

Repetitive DNA sequences including tandem repeats, satellites, ribosomal DNAs (rDNAs), and transposable elements (TEs), represent a large fraction of a eukaryotic genome (Biscotti et al. 2015). Such elements can be found at particular chromosomal loci or dispersed and are significant for understanding genome organization, structure, and function (Nandi et al. 2007; de Souza et al. 2013).

Among tandemly repeated sequences, rRNA belongs to multigene families encoding rRNAs 45S (18S-5.8S-26S) and 5S, which are responsible for the formation of ribosomal subunits. The distribution of microsatellites (simple sequence repeats [SSRs]; 2–5 bp), differs between genomes (Reilly et al. 1996; Schmidt and Heslop-Harrison 1996; Mesquita et al. 2003; Chistiakov et al. 2006), and their copy number variants can be used as genetic markers. SSRs may also play an important role in genome evolution and can be related with sex chromosomes in fishes (Ziemniczak et al. 2014) and to either coding or noncoding genomic regions (Cuadrado and Schwarzacher 1998; Tóth et al. 2000). TEs can be subdivided into transposons

and retrotransposons based on their transposition mode, with RNA as an intermediate in retrotransposons (Wicker et al. 2007). Increased rates of recombination can lead to greater probabilities of TEs establishing in populations (Groth and Blumenstiel 2016). The role of these elements has been discussed in fish and other aquatic organisms (Benjamin et al. 2007; Jiang et al. 2011; Barbosa et al. 2014). The Tc1-mariner transposon is defined by characteristic transposase gene domains, target site duplications (TSDs), and terminal inverted repeats (TIRs) (Muñoz-López and García-Pérez 2010; Menzel et al. 2014) and includes autonomous and nonautonomous elements. Nonautonomous elements do not encode the proteins required for their mobility, so they rely on autonomous elements, which encode all enzymes necessary for transposition (Wicker et al. 2007). Tc1/mariner elements are highly capable of invading a wide range of species by horizontal transfer (HT), because these are not dependent on host factors to mediate their mobility (Schaack et al. 2010; Zhang et al. 2016). Cases of HT events involving transposons between vertebrates and their parasites have been reported (Kuraku et al. 2012; Walsh et al. 2013; Zhang et al. 2014; Suh et al. 2016; Zhang et al. 2016). Fishes in the genus Imparfinis belong to the Neotropical catfish Heptapteridae family. They are considered one of the most diverse genera in the group, with 18 species (Reis et al. 2003), with variable karyotypes from 2n = 42 in Imparfinis hollandi (Margarido and Moreira-Filho 2008) to 2n = 58 in Imparfinis schubarti, (Kantek et al. 2009; Gouveia et al. 2013). The physical mapping of 18S, 5.8S, and 28S rDNA loci has given some insight about karyotype evolution and diversification in Imparfinis (Gouveia et al. 2013; Gouveia et al. 2016). Nucleolar organizer regions (NORs) are situated in the genomes of the most species in the Imparfinis genus with an interstitial location in a metacentric chromosome (Borba et al. 2012; Gouveia et al. 2013). Only I. hollandi has terminal NORs in subtelocentric chromosomes (Margarido and Moreira-Filho 2008) and Imparfinis borodini (referred to as Heptapterus longicauda) has multiple subterminal NORs (Vissotto et al. 1999). Sites of 5S rDNA in genomes of Imparfinis may be either on the same chromosome, as the 45S rDNA (Kantek et al. 2009; Ferreira et al. 2014), or located on different chromosomes and usually interspersed with heterochromatin blocks (Gouveia et al. 2013; Ferreira et al. 2014; Gouveia et al. 2016). Here, we isolated and characterized Tc1-mariner sequences in the I. schubarti genome, and mapped their locations with respect to rDNA and multiple microsatellite sequences in individuals I. borodini and I. schubarti from Brazilian river basins. The results will contribute to an understanding of the variability and evolution of these elements

Materials and Methods

Individuals Analyzed and Chromosome Banding Imparfinis schubarti and

1 I. borodini were collected from tributaries of the Paranapanema River and Ivaí River
2 basins, respectively (Figure 1 and Table 1). The samples were collected with permission
3 of the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
4 (IBAMA), under protocol number 11399-1. Specimens were deposited in the Museum
5 of Zoology of the University Estadual of Londrina (MZUEL), Parana, Brazil, with
6 voucher numbers: I. borodini: Pereira River (10480) I. schubarti: Água das Araras
7 River (10481); Quexada River (5763); Taquari River (10483), and Vermelho River
8 (10482). Number of animal ethic use in research ethics committee of the University
9 Estadual of Londrina: CEUA 28520.2012.03.

10 Mitotic chromosomes were obtained by direct preparation after removal of the
11 posterior kidney as described by Bertollo et al. (1978). The chromosomes were
12 organized according to Levan et al. (1964), with modifications, to determine the
13 fundamental number (FN; the number of chromosome arms in the complement).
14 Metacentric (m), submetacentric (sm) and subtelocentric (st) chromosomes were
15 considered biarmed, and acrocentric (a) chromosomes were considered uni-armed. The
16 distribution of heterochromatin was analyzed by Giemsa C-banding (Sumner 1972).
17 Silver nitrate staining of the active nucleolar organizer regions (Ag-NOR) was
18 performed according to the method of Howel and Black (1980). The GC bands were
19 detected with chromomycin A_3 (CMA_3) according to the method of Schweizer (1980).
20

21 Isolation of Tc1-mariner elements from *Imparfinis*

22 Genomic DNA from *I. schubarti* was extracted from the muscles using a
23 standard phenol/chloroform procedure (Sambrook and Russel 2001). For
24 characterization of the transposable element Tc1-mariner, Tss IR primer was used with
25 only one sequence (5'-TACAGTTGAAGTCGGAAGTTTACATAC-3'), according to
26 Nandi et al. (2007). PCR reactions were performed using a total of 50µL with 0.4 µM
27 primer, 0.16 mM dNTPs, 2.5 mM $MgCl_2$, 1U of Taq polymerase in 1x reaction buffer
28 (YorkBio) and 70 ng of the genomic DNA of the *I. schubarti*. The PCR cycle Tc1 was
29 94 °C for 30s, 55 °C for 1 min, and 72 for 2 min for 35 cycles. PCR products were
30 separated by electrophoresis, isolated, cloned and sequenced. Amplification of the
31 transposable element Tc1- mariner was confirmed using CENSOR (Kohany et al.
32 2006), Repbase and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.CGI) (Altschul et al.
33 1997). For identification of protein domains the HMMER web interface
34 (http://www.ebi.ac.uk/Tools/hmmer/) with the Pfam database (Finn et al. 2015). To

identify the terminal inverted repeat (TIRs), dotplots were used (Sonnhammer and Durbin 1995). Annotated sequences were deposited in the NCBI database (KU198862, KU198863 and KU198864).

Fluorescent in situ hybridization (FISH)

Fluorescent *in situ* hybridization was performed according to Pinkel et al. (1986) with modifications. The 18S rDNA probe used was the *Prochilodus argenteus* (Hatanaka and Galetti 2004), and 5S rDNA of the *I. schubarti* was isolated by Gouveia et al. (2016). Probes of synthetic microsatellite sequences were hybridized according Schmidt and Heslop-Harrison (1996) and oligonucleotides used were: $(GACA)_4$, $(GAA)_7$, $(CAC)_5$ and $(CA)_8$. Probes were labeled with Dig-nick translation Kit (Roche) or BioNick TM Labeling System Kit (Invitrogen). Preparations were covered with 50 μL of hybridization mixture containing 100 ng of labeled probe (7.5 μL), 50% formamide (30 μL), dextran sulfate 50% (12 μL) and 20x SSC (10.5 μL). The preparations were denatured at 80 °C for 10 min, and hybrided overnight at 37°C in a humidified chamber. Post-hybridization washes were carried out in 2x SSC for 5 min, in 1x PBS and 1x (20x SSC, Triton 100, non-fat milk and distilled water, pH 7), all at 45°C. The probe was detected with 5 μL of avidin conjugated with FITC (1:100) and anti-digoxigenin with rhodamine conjugate + 45 μL of BSA (5%) as appropriate. To amplify the signal, 40 μl of amplification solution (1 μL anti-avidin-biotin conjugate and 39 μL of 1x (20x SSC, Triton 100, non-fat milk and distilled water, pH 7) were used. The slides were mounted with 25 μL of a medium composed of 23 μL of DABCO solution (1, 4-diaza- bicyclo (2.2.2)-octane (2, 3%), 20 mM TrisHCl, pH 8.0, and glycerol (100%), in distilled water), 1 μL of $MgCl_2$ 50 mM and 1 μL of DAPI solution (20 μg/mL). Images were acquired with a Leica DM 4500 B or Nikon E800 microscopes. Images were overlaid and processed in Adobe Photoshop using only cropping and functions affecting the whole image equally.

Results

The C-banding, CMA3 , and FISH using 18S and 5S rDNA probes on chromosomes of 2 Imparfinis species showed differences in their karyotypes. Imparfinis schubarti had 2n = 58 and FN = 116 and karyotype with 30 m + 28 sm chromosomes in the 4 populations studied (Figure 2a). A secondary constriction was detected in the

interstitial region of the q arm of metacentric pair 1, coincident with the Ag-NOR, CMA3 , and 18S rDNA positive sites (Figure 2a box and Figure 5a, c). The 5S rDNA site was located in the region close to the centromere on metacentric pair 10, which also showed CMA3 bands in this region (Figure 2a box). Imparfinis borodini had 2n = 50 and FN = 100 with a karyotype with 24 m + 18 sm + 8 st. The chromosomes of the first pair were larger in size compared with the other chromosomes of the complement (Figure 2c). The Ag-NOR was in the terminal region of the p arm of metacentric pair 6, confirmed by CMA3 and the 18S rDNA positive sites (Figure 2c box). The 5S rDNA cistrons were located proximally to 18S rDNA, in the interstitial region of the p arm of pair 6 (Figure 2c box and Figure 5c). C-banding showed bands in the pericentromeric, interstitial, and terminal regions of most chromosomes in I. schubarti, with a large interstitial block at the 18S rDNA site on chromosome No . 1 (Figure 2b and Figure 5a, c); pair 10 also showed a large heterochromatic block in the interstitial region corresponding with the 5S rDNA site (Figure 2b). Chromosomes of I. borodini had interstitial and terminal blocks in some chromosomes as well as a block in the terminal region of the p arm of pair 6 coincident with the Ag-NOR (Figure 2d).

Characterization of Tc1-Mariner in the *I. schubarti* Genome and its Genomic Location in *I. borodini* and *I. schubarti*

The Tc1-mariner primers amplified products of 800bp, 600bp, and 300bp from *I. schubarti* genomic DNA with similarity to Tc1_TF and Mariner-1B_EL, both belonging to DNA/Mariner non-autonomous classes in all sequences (Censor; supplementary material S1). The HMMER program identified several similarities with the helix-turn-helix (HTH) in the 300bp sequence and in the 600bp sequence two domains were detected: homeodomain-like domain (HLD) and helix-turn-domain helix (HTH) (supplementary material S2). However, no ORF (open reading frame) encoding a complete transposase was detected in any sequence. Dotplots identified the TIR (terminal inverted repeat) of the 800bp sequence was about 27 nucleotides long from 1 to 27bp and 795 to 822bp, with TA dinucleotides in both TIRs. In the 600bp sequence, the TIRs with 23bp were detected in the 5' direction. In the 300bp sequence with TA dinucleotides, the TIRs were identified in the same direction.

Hybridization with the Tc1-mariner transposon showed discrete locations in the terminal and pericentromeric regions of most chromosomes *I. schubarti*, coincident with the distribution of heterochromatin in this species. Some pairs showed bitelomeric

1 markings, e.g., NOR pair 1 and 5S rDNA pair 10 (Fig. 3a, 5a). In *I. borodini,*
2 hybridization with Tc1 appeared in conspicuous blocks in some chromosomes,
3 coincident with mostly interstitial C-banded heterochromatin (Fig. 3b). Subtelocentric
4 chromosomes showed terminal sites on the short arm (24 and 25) (Fig. 3b) and pair 6
5 (with 18S and 5S rDNAs) had a large Tc1-mariner block in the interstitial region (Fig.
6 2d and 3b). In the *I. schubarti* interphase nucleus, this transposon appeared dispersed in
7 the nucleus; in *I. borodini* it appeared in isolated blocks (Fig. 3a and b-box
8 respectively).
9
10 Microsatellite (simple sequences repeat) locations
11 The simple microsatellite sequences $(GAA)_7$, $(GACA)_4$, $(CAC)_5$, and $(CA)_8$
12 showed different and characteristic distribution patterns in the genome of *I. schubarti*
13 and *I. borodini.* $(GAA)_7$ displayed discrete and dispersed markings on almost all
14 chromosomes of both species; and it was abundant on pair 1, especially in *I. borodini*
15 (Fig. 4b-arrows and Fig. 5b). We could observe more specific locations on a
16 chromosome of *I. schubarti* with an interstitial band on pair 1 (Fig. 4a-arrows and Fig.
17 5a). $(GACA)_4$ showed dispersal on all chromosomes, with small terminal and interstitial
18 points, and pair 1 of *I. borodini* showed an accumulation in the interstitial region (Fig.
19 4d-arrows and Fig. 5b), coinciding with the heterochromatin block (Fig. 2d and Fig.
20 5b). Also, pair 1 of *I. schubarti* showed interstitial locations at the constriction region
21 (Fig. 4c and Fig. 5a).
22 Microsatellite (CAC)5 appeared to be distributed as a block in the centromeric
23 regions of some chromosomes of the 2 species (Figure 4e, f). Pair 1 of *I. borodini*
24 revealed an interstitial block, coincident with the C-band and a small block in the
25 interstitial region of the q arm (Figure 4f arrows and Figure 5b). Pair 1 of *I. schubarti*
26 exhibited a large block coincident with the secondary constriction (Figure 4e arrows and
27 Figure 5a).
28 Only a few *I. borodini* and *I. schubarti* chromosomes had signals with
29 microsatellite repeats (CA)8 in the interstitial and terminal positions. Pair 1 of
30 I. schubarti had a more evident signal in the interstitial region (Figure 4g arrows and
31 Figure 5a) and pair 1 of I. borodini had a discreet signal in the bitelomeric region and a
32 conspicuous mark in the interstitial position (Figure 4h arrows and Figure 5b)
33

1 **Discussion**

2 The diploid number was 2n=58 in *Imparfinis schubarti,* as found in other
3 populations of this species (Kantek et al. 2009; Borba et al. 2012; Gouveia et al. 2013;
4 Gouveia et al. 2016). For *I. borodini*, 2n = 50 is a new report for the species,
5 corroborating the karyotype variability existing within the genus. Another population of
6 *I. borodini* from the upper Paraná basin analyzed by Vissotto et al. (1999) was 2n = 52
7 with multiple NORs on the long arm of pairs 2 and 25, differing from the population
8 here, with a terminal NOR on the short arm of pair 6. Notably, the first chromosome
9 pair, much larger than the other pairs of the complement, was variable and serves as a
10 chromosomal marker for this population. This pair 1 also has accumulation of several
11 microsatellite repeats (Fig. 5b). Microsatellite accumulation can be related to several
12 aspects of behavior of the genome of eukaryotes including chromatin organization (Li et
13 al. 2002).

14 Margarido and Moreira-Filho (2008) suggest that the evidence of asymmetric
15 chromosomes in the karyotype of another species of the genus *Imparfinis* (*I. hollandi*),
16 can be an indication of chromosome fusions. In the mammalian Bovideae group,
17 karyotype variability both within and between species involves fusion of acrocentric
18 chromosomes, so the number of autosomal arms remains similar (Chaves et al. 2003).
19 Despite the cytogenetic diversity within the Heptapteridae family, Borba et al. (2012)
20 found that there is a prevalence of 2n = 58 with the presence of biarmed chromosomes.
21 Thus 2n = 58 is considered a basal plesiomorphic feature in the family (Fenocchio et al.
22 2003) and the reduction of the number of chromosomes in some species of *Imparfinis* is
23 a synapomorphic condition in the genus (Borba et al. 2012; Ferreira et al. 2014).

24 The interstitial NOR location is also a synapomorphic characteristic of the
25 family Heptapteridae since most other species have terminal NORs (Kantek et al. 2009;
26 Borba et al. 2012). *I. borodini* has a terminal NOR on the short arm of a pair of small
27 metacentric chromosomes (pair 6); we support the hypothesis that inversion and
28 duplication events led to the derived interstitial NOR positions in the karyotype (Borba
29 et al. 2012). Chromosome 1 of *I. borodini* and *I. schubarti* evidently accumulates
30 different repetitive DNA sequences (Fig. 5). Pair 1, characteristic of *I. schubarti,* is
31 similar in other species with an interstitial NOR site (*I. mirini, I. minutes,* and *I.*
32 *piperatus*; review tables: Borba et al. 2012; Yano and Margarido 2012; Gouveia et al.
33 2013; Ferreira et al. 2014, and the present study). However, pair 1 of *I. borodini* does

1 not have any similarity with other species of this genus, and can be considered a marker
2 pair.

3 Transposons are present in both telomeric regions of pair 1 of *I. schubarti* (18S
4 rDNA) (Fig. 5c). Tc1-Mariner is also abundant in the chromosomes with 5S rDNA sites
5 in both species. These TEs thus correlate with inversion in the NOR pair or 5S rDNA
6 gene transposition, and alongside this information the similarity of 5S rDNA of *I.*
7 *schubarti* with SINE3-1 (see Gouveia et al. 2016), suggest that transposons may be
8 playing an important role in the transposition of 5S rDNA in the species of this genus,
9 thus corroborating to dispersion and evolution of these sequences as seen in another fish
10 genomes (Costa et al. 2013; Yano et al. 2014).

11 In *Chionodraco hamatus*, the Tc1 transposon also accumulated at
12 heterochromatic regions, suggesting that this element can be associated with
13 chromosome rearrangements in fish (Capriglione et al. 2000). The Tc1 transposon
14 sequences here were degenerate and non-autonomous, along with the related to
15 Miniature Inverted–Repeat Transposable Elements (MITEs) in eukaryotes (Feschotte et
16 al. 2002a). Tc1 is coincident with heterochromatin blocks (chromocentres), perhaps
17 related to inactivation (for review, see Feschotte 2008) and also control of chromatin
18 packaging by these in *Imparfinis*. Notably, the Tc1-mariner elements here differed in
19 their dispersion pattern in interphase nuclei of two species of *Imparfinis*. In this case
20 Tc1 is probably similar to heterochromatic distribution in nuclei of both species. In
21 plant analysis by FISH on hypomethylated nuclei revealed that high-copy transposon
22 elements from different families, including miniature inverted-repeat transposable
23 element (MITE), still co-localize within chromocenters nuclei and it is important that
24 transposon elements are also anchor points for formation of the heterochromatin
25 through of DNA methylation (Fransz et al. 2003).

26 Active Tc1-mariner have been reported, with complete transposases in the
27 Japanese fish Medaka (*Oryzias latipes*) (Koga and Hori 2000); Parodontidae fish this
28 transposon is important to potential participation in the differentiation processes of sex
29 chromosomes (Schemberger et al. 2016) and in *Danio rerio* (zebrafish) Tol2-Tyr in the
30 Tc1-mariner family, encoding a functional transposase catalyzing transposition in the
31 Zebrafish germ lineage (Kawakami et al. 2000). Because of Tc1-mariner sequences
32 isolated in this study have incomplete binding domains with low similarity to each
33 other; we suggest that insertion and mutation events could have led to molecular
34 deterioration of this element in *Imparfinis* genomes. This deterioration process where

TEs may incorporate different classes of errors of different types, according to their transposition mechanism (Fernández- Medina et al. 2012; Schemberger et al. 2016) are expected within genomes.

Microsatellite sequences showed characteristic and different distribution patterns in *I. borodini* and *I. schubarti*. Some were dispersed in the genome [microsatellites $(GACA)_4$ and $(GAA)_7$], while others accumulated in terminal and interstitial regions [$(CAC)_5$, $(CA)_8$], perhaps reflecting the age of dispersion in the fish genome, as suggested by Yano et al. (2014). Vanzela et al. (2002) also showed $(GA)_9$+C microsatellite sites in terminal regions of chromosomes of another population of *I. schubarti.*

In conclusion, the genus and even species within *Imparfinis* show high levels of variability in karyotype and repetitive DNA organization (18S and 5S rDNA, transposable elements and microsatellites). Clearly, some repeats are accumulating in heterochromatic regions (as noted in other fish: Ferreira and Martins 2008; Cioffi et al. 2010; Poltronieri et al. 2014) where they may have a role related to both genome activation and chromatin packaging; or to chromosomal rearrangement through fissions and fusions. Repetitive DNA accumulation may enable and lead to the variable chromosome numbers seen even within a single species. Physical mapping of repeats is helpful for structural genomics studies and fish genetics: here we can relate the changes in organization to the divergence of karyotypes between *I. borodini* and *I. schubarti* through building a picture of their chromosome evolution.

Conflict of interest

The authors have no conflicts of interest to declare.

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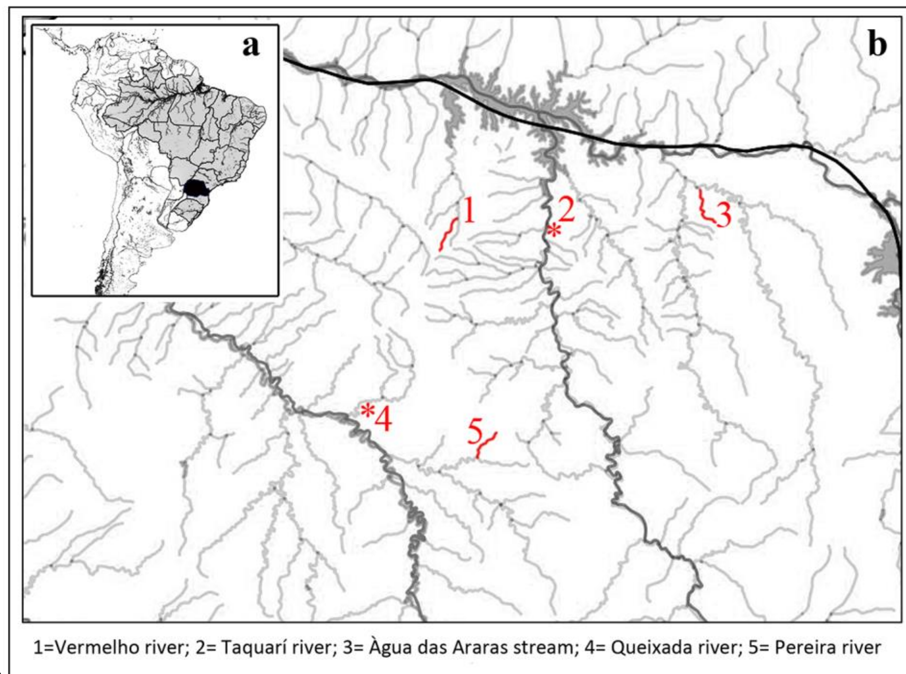

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Legends

Fig. 1. Map of Brazil indicating the collection sites: (a) South America with (black) Paraná state. (b) The rivers tributaries of the Paranapanema River basin (1, 2 and 3) and the Ivaí River basin (4 and 5). Scale: 10 km.

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Fig. 2. Feulgen-stained karyotype of *Imparfinis schubarti* (a) and *Imparfinis borodini* (c) with evidence of asymmetry in chromosome pair 1 (c). Inset box shows location of AgNOR with silver nitrate; staining with CMA_3; and in situ hybridization locating 18S rDNA probe and 5S rDNA. C-banded karyotypes of *I. schubarti* (b) and *I. borodini* (d) show heterochromatic blocks on some chromosome pairs. Bar = 5µm.

Fig. 3. Karyotype of (a) *Imparfinis schubarti* and (b) *Imparfinis borodini* with fluorescence *in situ* hybridization showing the distribution of Tc1-mariner probe. Inset: interphase nuclei with contrasting distributions of Tc1-mariner. Bar = 5µm.

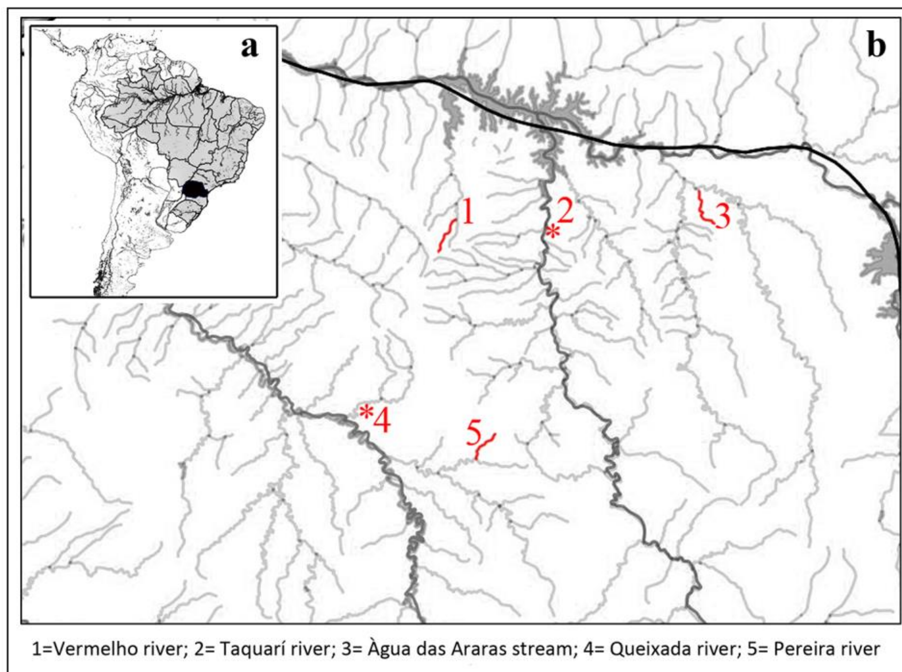
Fig.4. Somatic metaphases of the *Imparfinis schubarti* (a, c, e, g) and *Imparfinis borodini* (b, d, f, h), showing the fluorescence *in situ* hybridization with sintetic microsatellite probes. (a, b) with microsatellite (GAA) 7; (c, d) microsatellite (GACA) 4; (e, f) microsatellite (CAC) 5 and (g, h) microsatellite (CA) 8. Bar = 5µm.

Fig. 5. Representative ideogram of pair 1 of *Imparfinis schubarti* (a) and *Imparfinis borodini* (b) showed accumulation of different repetitive DNAs with cytogenetic markers (C-banding, NOR, CMA_3, Mariner and microsatellites (GAA, GACA, CAC and CA)). In (c) pairs 1 and 10 of *I. schubarti* and pair 6 de *I. borodini*, with rDNA 18S and 5S sites and distribution of Tc1-mariner in both species.

Supplementary Material

Supplementary Material S1-pdf- Results of CENSOR software shows in sequences with 800bp, 600bp and 300bp isolated from *Imparfinis schubarti* genome with the similarity with Tc1-mariner.

Supplementary Material S2-pdf- Results of HMMER interface with identification of protein domains helix-turn-helix (HTH) in sequence with 300bp and 600bp of the transposon Tc1-mariner from *Imparfinis schubarti* genome.



1=Vermelho river; 2= Taquarí river; 3= Àgua das Araras stream; 4= Queixada river; 5= Pereira river

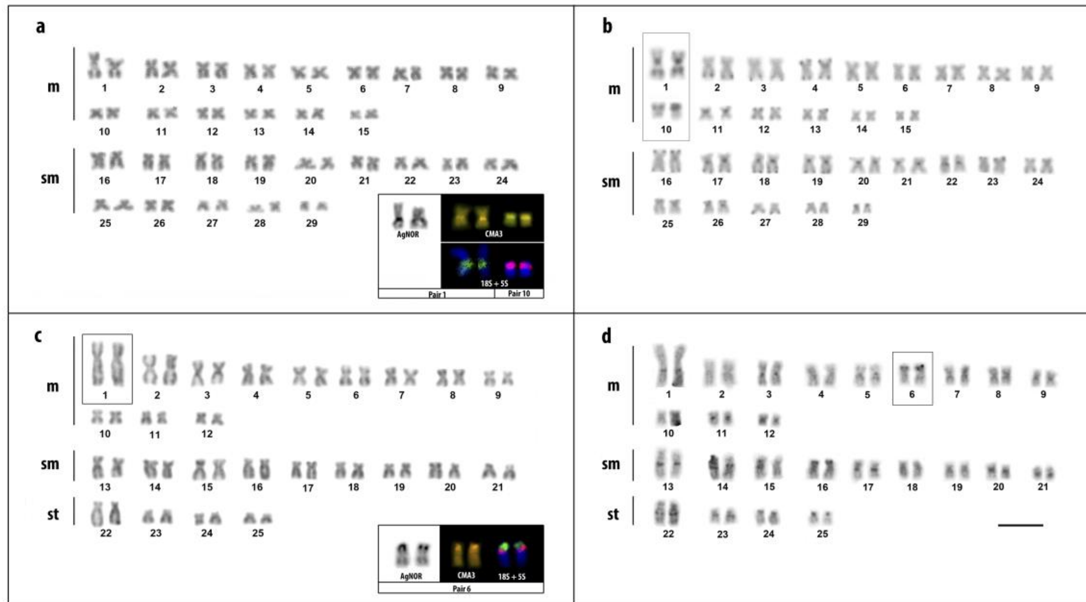
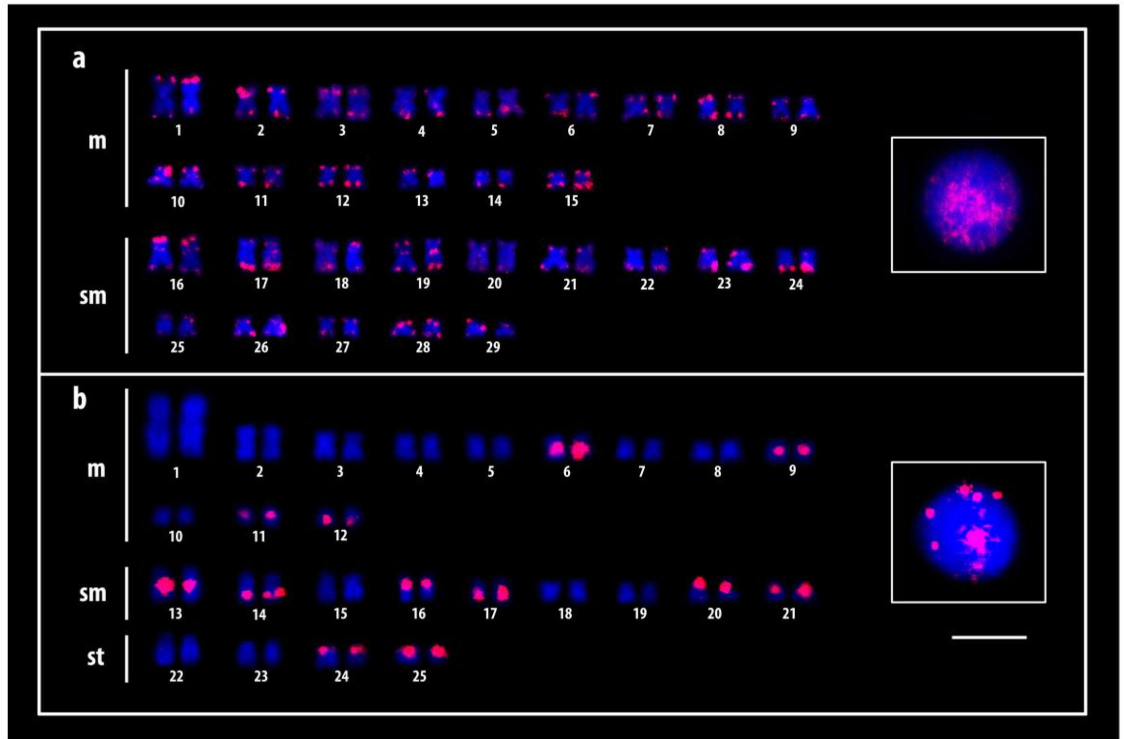


Figure 2.



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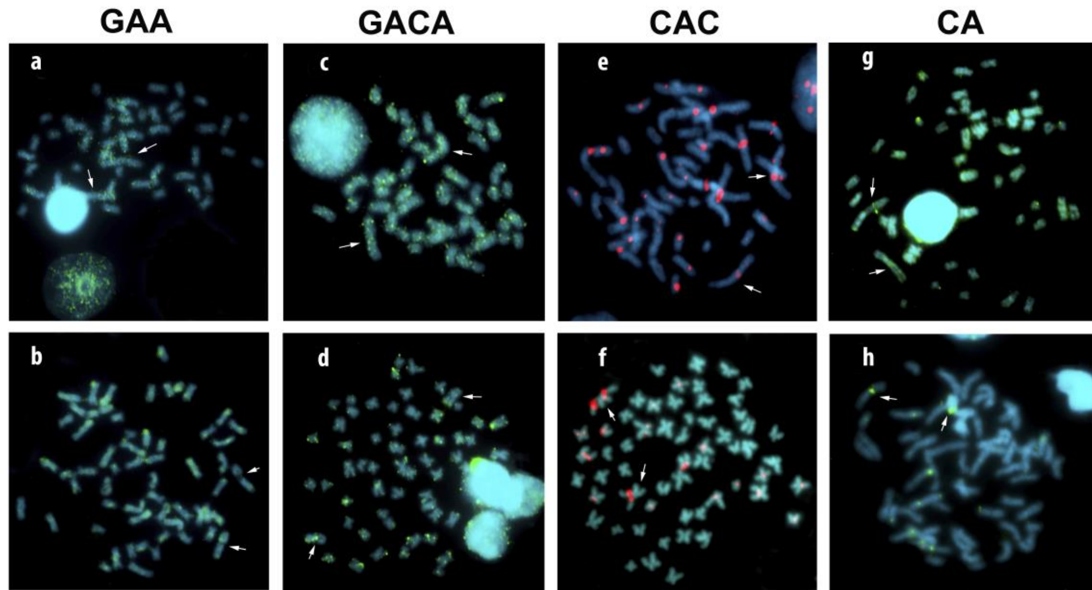

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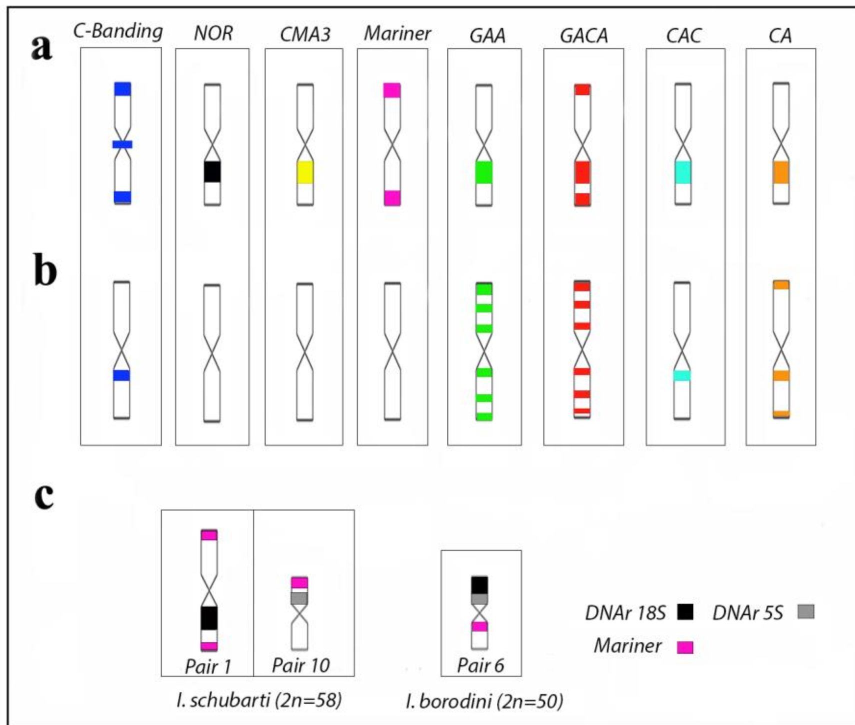

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2 Figure 5.