



Evolutionary Dynamics of Multigene Families in *Triportheus* (Characiformes, Triporthidae): A Transposon Mediated Mechanism?

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Triportheus (Characiformes, Triporthidae) is a freshwater fish genus with 18 valid species. These fishes are widely distributed in the major river drainages of South America, having commercial importance in the fishing market, mainly in the Amazon basin. This genus has diverged recently in a complex process of speciation carried out in different river basins. The use of repetitive sequences is suitable to trace the genomic reorganizations occurred along the speciation process. In this work, the 5S rDNA multigene family has been characterized at molecular and phylogenetic level. The results showed that other multigene family has been found within the non-transcribed spacer (NTS): the U1 snRNA gene. Double-FISH with 5S and U1 probes were also performed, confirming the close linkage between these two multigene families. Moreover, evidences of different transposable elements (TE) were detected within the spacer, thus suggesting a transposon-mediated mechanism of 5S-U1 evolutionary pathway in this genus. Phylogenetic analysis demonstrated a species-specific grouping, except for *Triportheus pantanensis*, *Triportheus* aff. *rotundatus* and *Triportheus trifurcatus*. The evolutionary model of the 5S rDNA in *Triportheus* species has been discussed. In addition, the results suggest new clues for the speciation and evolutionary trend in these species, which could be suitable to use in other Characiformes species.

Keywords: *Triportheus*, 5S rRNA, U1 snRNA, transposable elements, speciation

INTRODUCTION

Multigene families correspond to a group of genes that have originated by duplication of a common ancestral gene and therefore have similar DNA sequences and related overlapping functions (Martins and Wasko, 2004; Nei and Rooney, 2005). The 5S rDNA array is characterized by multiple copies of one conserved transcriptional unit of about 120 base pairs, separated from each other by a non-transcribed spacer (NTS), which is variable in length and sequence between species (Rebordinos et al., 2013). The NTSs have been subjected by intense mechanisms of evolution, allowing a broader understanding of the organization and evolution of the 5S rDNA array in many organisms (Martins and Wasko, 2004). Additionally, the 5S rDNA can be found linked to other

multigene families, including major ribosomal genes, histones, and small nuclear RNA (snRNA) genes (Drouin and Moniz de Sá, 1995; Machado et al., 2006; Cabral-de-Mello et al., 2010).

The levels of intra or intergenomic divergence displayed by 5S rRNA genes have led the discussion of different evolutionary models to explain the evolution pattern found in each species or between species. A vast number of studies have pointed that the concerted evolution has driven the evolution of 5S rDNA and other multigene families (Drouin and Moniz de Sá, 1995; Eirín-López et al., 2012). However, further studies have demonstrated that the concerted evolution does not clarify the intragenomic divergence of several multigene families found in some organisms, and another model called birth-and-death was proposed (Nei and Hughes, 1992). Besides, a growing number of studies have combined these both models to illustrate the diversified scenarios regarding the 5S rDNAs evolution (Merlo et al., 2012a, 2013). Therefore, NTS regions seem to be subject to rapid evolution, which makes them important for studies concerning the organization and evolution of the 5S multigene family, and as markers for species-specific studies or tracing recent evolutionary events (Rebordinos et al., 2013).

Triportheus (Characiformes, Triporthidae) is a freshwater fish genus with 18 valid species (Froese and Pauly, 2019). These fishes are widely distributed in the major river drainages of South America, having commercial importance in the fishing market, mainly in the Amazon basin (Malabarba, 2004; Gonçalves and Batista, 2008). According to a recent phylogenetic study established for this genus, *Triportheus* originated at 26.2 ± 6.5 Myr and represents a monophyletic group in which *T. auritus* is the oldest living species within the genus (Mariguela et al., 2016). *Triportheus* has stood out in many cytogenetics studies, mainly because all species studied so far have a ZW sex chromosomes system well characterized (Artoni et al., 2001; Diniz et al., 2009; Yano et al., 2016, 2017a). In addition, the rDNAs (Diniz et al., 2009; Marquioni et al., 2013; Yano et al., 2017b) and the U2 snRNA genes (Yano et al., 2017b) have been also investigated, highlighting the variability in the syntenic configuration as well as in the number of sites of these multigene families, mainly regarding the 5S rDNAs (Yano et al., 2017b).

Large volumes of studies have been published on physical mapping of rDNAs in divergent taxa, especially in fish. However, the integration of cytogenetic data with molecular analysis has increasingly brought insights about the evolutionary mechanisms of multigenic families (Machado et al., 2006; Cabral-de-Mello et al., 2012; Merlo et al., 2012b, 2013). Hence, we have investigated the 5S rRNA genes by molecular and cytogenetics analyses in eight *Triportheus* species in order to better understand the organization and the evolutionary pattern of this multigene family within the genus. We described here a repeat unit containing the 5S rRNA gene linked to U1 snRNA gene, and therefore, the double-FISH technique was applied to ascertain the results found in the sequences analyses. Finally, the nucleotide variability and the evolutionary divergence between species were estimated to assess if the concerted evolution or birth and death, or if both models are driving the 5S rDNAs in *Triportheus*.

MATERIALS AND METHODS

Material Collection, Chromosome Obtaining and DNA Extraction

Individuals of *Triportheus albus*, *Triportheus auritus*, *Triportheus guentheri*, *Triportheus nematurus*, *Triportheus pantanensis*, *Triportheus aff. rotundatus*, *Triportheus signatus*, and *Triportheus trifurcatus* from different Brazilian river basins were analyzed. The collections were authorized by the Brazilian environmental agency ICMBIO/SISBIO (License number 48628-2). All species were identified and deposited in the fish museum of the Laboratory of Biology and Genetic of Fishes of the Universidade Estadual Paulista (UNESP-Botucatu, SP), with the respective deposit numbers (Table 1). The experiments followed ethical conducts, in accordance with the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (Process number CEUA 1853260315). Mitotic chromosomes were obtained from cell suspensions of the anterior kidney using the conventional air-drying method, according to Bertollo et al. (2015). Genomic DNA was isolated from liver, following the protocol of Sambrook and Russel (2001), and the extraction quality was validated by electrophoresis in agarose gel (1.5%), using GelRed in the loading buffer.

5S rDNA Isolation, Cloning, and Sequence Analysis

The PCR amplifications of the 5S rDNA were performed using the following primers 5SF (5'-TACGCCGATCTCGTCCGATC-3') and 5SR (5'-CAGGCTGGTATGGCCGTAAGC-3'), as described in Pendas et al. (1994). Reactions were carried out using 0.2 pmol of the forward and reverse primers, 100 ng of genomic DNA, 3 mM Cl₂Mg, 300 μM dNTP and 3 U of Taq polymerase (Euroclone) in a final volume of 50 μl. The PCR amplification reactions were performed in a Gene Amp_PCR System 2700 (Applied Biosystems) thermal cycler, according to Cross et al. (2005). The PCR products were purified applying the NucleoSpin® Extract II kit (Macherey-Nagel), cloned into pGEM®-T Easy (Promega) and TOPO® TA Vectors, and the plasmid DNA was extracted using NucleoSpin® Plasmid (Macherey-Nagel), all of these kits were used under the manufacturer's instructions. The positive clones were sequenced using an ABI3100 Genetic Analyzer, with fluorescence-labeled terminator (BigDye Terminator 3.1 Cycle Sequencing Kit; Applied Biosystems).

Sequence and Phylogenetic Analysis

Sixty-six 5S rDNA-bearing clones were sequenced in this study, and the number of clones for species and individual are described in Table 2. The consensus sequences were obtained with the Geneious software (Kearse et al., 2012), and were subjected to BLASTn searches at the National Center for Biotechnology Information website¹ – NCBI. In addition, the CENSOR program (Kohany et al., 2006) was used to search for repeated elements inside spacers. The sequence alignments were performed using

¹<http://www.ncbi.nlm.nih.gov/blast>

TABLE 1 | Brazilian collection sites of the *Triporthesus* species and number of individuals and cells examined in this study.

Species	Site	Basin	N	Number of cells	Deposit number
<i>Triporthesus albus</i>	Araguaia river	Araguaia-Tocantins	04♂; 04♀	40	LBP18620
<i>Triporthesus auritus</i>	Araguaia river	Araguaia-Tocantins	05♂; 04♀	25	LBP18622
<i>Triporthesus guentheri</i>	Inhuma lake	São Francisco	12♂; 06♀	30	LBP18628
<i>Triporthesus nematurus</i>	Paraguai river	Paraguai	09♂; 07♀	20	LBP18624
<i>Triporthesus pantanensis</i>	Paraguai river	Paraguai	01♂; 01♀	30	LBP18623
<i>Triporthesus aff. rotundatus</i>	Paraguai river	Paraguai	19♂; 21♀	25	LBP18625
<i>Triporthesus signatus</i>	Piracicaba river	Tietê	13♂; 24♀	25	LBP18619
<i>Triporthesus trifurcatus</i>	Araguaia river	Araguaia-Tocantins	04♂; 11♀	20	LBP18621

MUSCLE (Edgar, 2004). Next, the sequences obtained in this study were deposited in the NCBI database under the Accession Numbers listed in **Table 2**. The nucleotide variability (π) within species, the number of polymorphic sites (s) and the number of haplotypes (h) in both transcribed and spacer regions were estimated by the DnaSP version 5 program (Librado and Rozas, 2009). The evolutionary divergence among species was also inferred using DnaSP, by the average number of nucleotide differences per site using the Jukes and Cantor method [Dxy(JC)], the number of fixed differences in transcribed region and NTS was also inferred. The PhyML 3.0 program (Guindon et al., 2010) was used to determine the best-fit phylogenetic model and then to run the model. The resulting best-fit model was the generalized time-reversible (GTR) model (Tavaré, 1986), considering the proportion of invariable sites (+I) and gamma distribution (+G). The statistic used for model selection was the akaike information criterion (AIC), the value of which was 18315.92230, and the $-\ln L$ was -8784.01056 . Branch support was tested by the fast likelihood-based method using aLRT SH-like (Anisimova et al., 2011). Finally, the tree was edited in the MEGA7 program (Kumar et al., 2016). Besides, 5S rDNA sequences were also subjected to a neighbor-net analysis (Bryant and Moulton, 2004) implemented in the SplitsTree 4 package (Huson and Bryant, 2006) using GTR distances.

Probe Preparation

The PCR product of 5S rDNA was labeled with Cy5-dUTP using Nick-Translation Mix (Roche). The U1 snDNA probe was isolated using the following primers U1F (5'-GCAGTCGAGATTCCCACATT-3') and U1R (5'-CTTACCTGGCAGGGGAGATA-3'), as described by

Silva et al. (2015). The U1 snRNA probe was labeled via PCR using the same set of primers and the cycle program described in Silva et al. (2015) with Spectrum Orange-dUTP (Vysis).

Fluorescence *in situ* Hybridization

Chromosomal preparations of *Triporthesus* species listed in **Table 1** were used for a double-color fluorescence *in situ* hybridization (FISH) experiment, according to Yano et al. (2017c). The slides were first dehydrated with 70, 85, and 100% ethanol series, and incubated at 60°C for 1 h. Subsequently, they were treated with RNase (10 mg/mL) for 1 h at 37°C. Next, a 5-min wash using 1× phosphate-buffered saline (PBS) was performed and the slides were treated with 0.005% pepsin solution in 10 mM HCl at 37°C. The slides were washed again with 1× PBS for 5 min. After further washing, the slides were dehydrated with 70, 85, and 100% ethanol series, 3 min each. The desaturation of the chromosomal DNA was performed in 70% formamide/2× SSC for 3 min at 75°C. The slides were dehydrated in a cold ethanol 70%, and in 85 and 100% at RT, 3 min each. The hybridization mixture, containing 100 ng of each probe, 10 mg/mL dextran sulfate, 2× SSC, and 50% formamide in a final volume of 20 μ L, was heated to 86°C for 10 min and then applied to the slides. Hybridization was performed during 16 h at 37°C. After hybridization, the slides were washed in 1× SSC for 5 min at 44°C, and in 4× SSCT using a shaker at room temperature. The slides were dehydrated again in an ethanol series, 3 min each. After the complete drying of the slides, the chromosomes were counterstained with 4',6-diamidino-2-phenylindole/antifade (1.2 mg/mL; Vector Laboratories).

Microscope Analyses and Image Processing

At least 30 metaphase spreads were analyzed per individual to confirm the FISH results. Images were captured on an Olympus BX50 microscope (Olympus Corporation, Ishikawa, Japan) using CoolSNAP and the Image Pro Plus 4.1 softwares (Media Cybernetics, Silver Spring, MD, United States).

RESULTS

5S rDNA Organization in *Triporthesus*

Electrophoresis of PCR amplifications of 5S rDNAs revealed fragments of \sim 1600 bp in length for the *Triporthesus* species.

TABLE 2 | Species, number of individuals (N), number of clones sequenced (NC), and GenBank accession number.

Species	N	NC	GenBank Acc. No.
<i>T. albus</i>	3	9	MN708408 to MN708416
<i>T. auritus</i>	3	8	MN708417 to MN708423
<i>T. guentheri</i>	3	8	MN708424 to MN708431
<i>T. nematurus</i>	3	9	MN708432 to MN708440
<i>T. pantanensis</i>	2	6	MN708441 to MN708446
<i>T. aff. rotundatus</i>	3	9	MN708447 to MN708455
<i>T. signatus</i>	3	8	MN708456 to MN708463
<i>T. trifurcatus</i>	3	9	MN708464 to MN708472

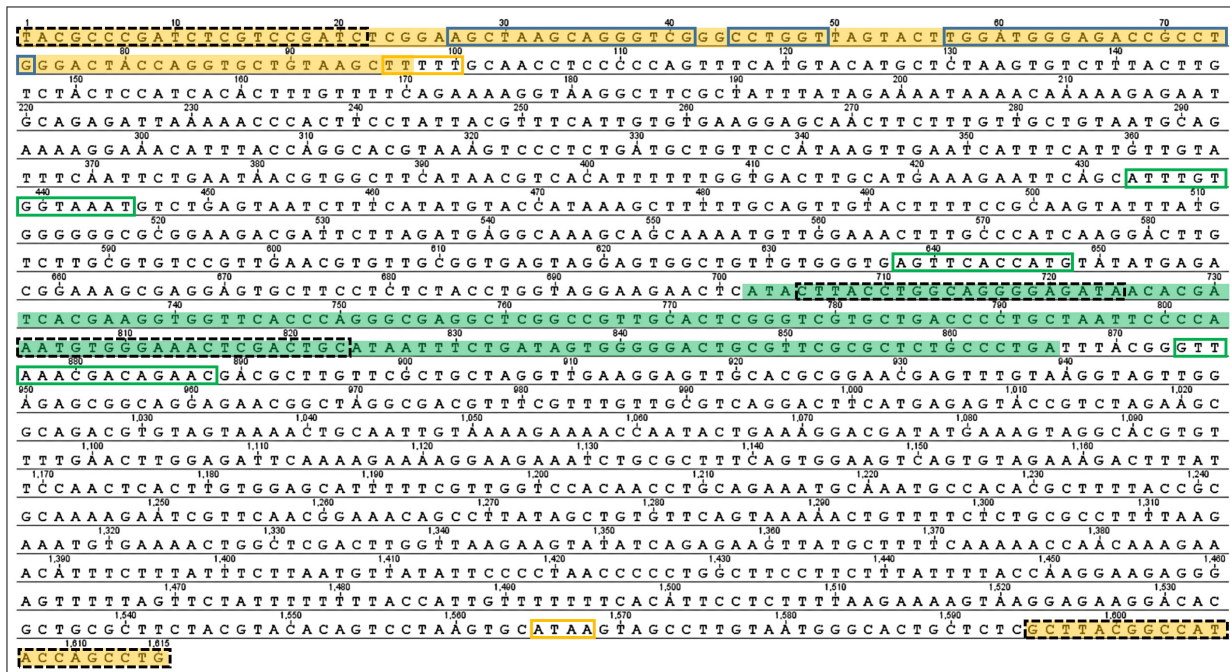


FIGURE 1 | Nucleotide sequence of the 5S rDNA from *Tripurtheus nematurus*. The sequence corresponding to the 5S rRNA transcribed region is highlighted in yellow. The U1 snRNAs gene is highlighted in green. Dash-lined boxes indicate the primer sequences. Blue boxes denote the Internal Control Regions of 5S rRNA (box A, intermediate element; box C, from left to right). The 5S rDNA external regulatory elements are boxed in yellow (poli-T termination region and TATA box, from up to down). The U1 snDNA regulatory elements are boxed in green (DSE, PSE, and 3' box, from up to down).

A total of 66 clones were sequenced in the present study, as detailed in **Table 2**. After using BLASTn, 65 sequences matched with the 5S rRNA gene, consisting in a conservative transcribed region of 120 bp, and an adjacent and variable NTS. The **Figure 1** shows an example of the 5S rDNA amplicon of *T. nematurus*, in which the transcribed region shows the internal control regions (ICR) for all species: Box A located at position 27–41 (positions 50–64 of the 120 bp-transcribed region); intermediate element (IE) at position 44–49 (positions 67–72 of the 120 bp-transcribed region); and Box C at position 57–74 (positions 80–97 of the 120 bp-transcribed region). The terminator region, a T-rich stretch at the end, was also verified and consists of 4–7 thymidine residues. In all sequences the TATA-like (conserved AT rich region) is located at position –30 within the NTS, the GC dinucleotide is at position –11, and the universally conserved cytosine at position –1.

The NTS has a peculiarity, since an U1 snRNA gene was found inside it at position 726–891 (**Figure 1**). The U1 snRNA sequences show identities of 95% with *Argyrosomus regius* and *Takifugu rubripes* fish species, and 92% with the frog *Xenopus laevis*. This gene divide the NTS in two fragments, one before the U1 gene (Spacer 1, at position 98–725) and other after U1 (Spacer 2, at position 892–1651 bp) (**Figure 1**). Therefore, 5S rRNA and U1 snRNA sequences are linked in the same array in all *Tripurtheus* species analyzed, and both genes displayed the same orientation. The regulatory regions were identified: (i) a proximal sequence element (PSE) is located at 56 nucleotides upstream of the transcription point; (ii) a distal sequence element

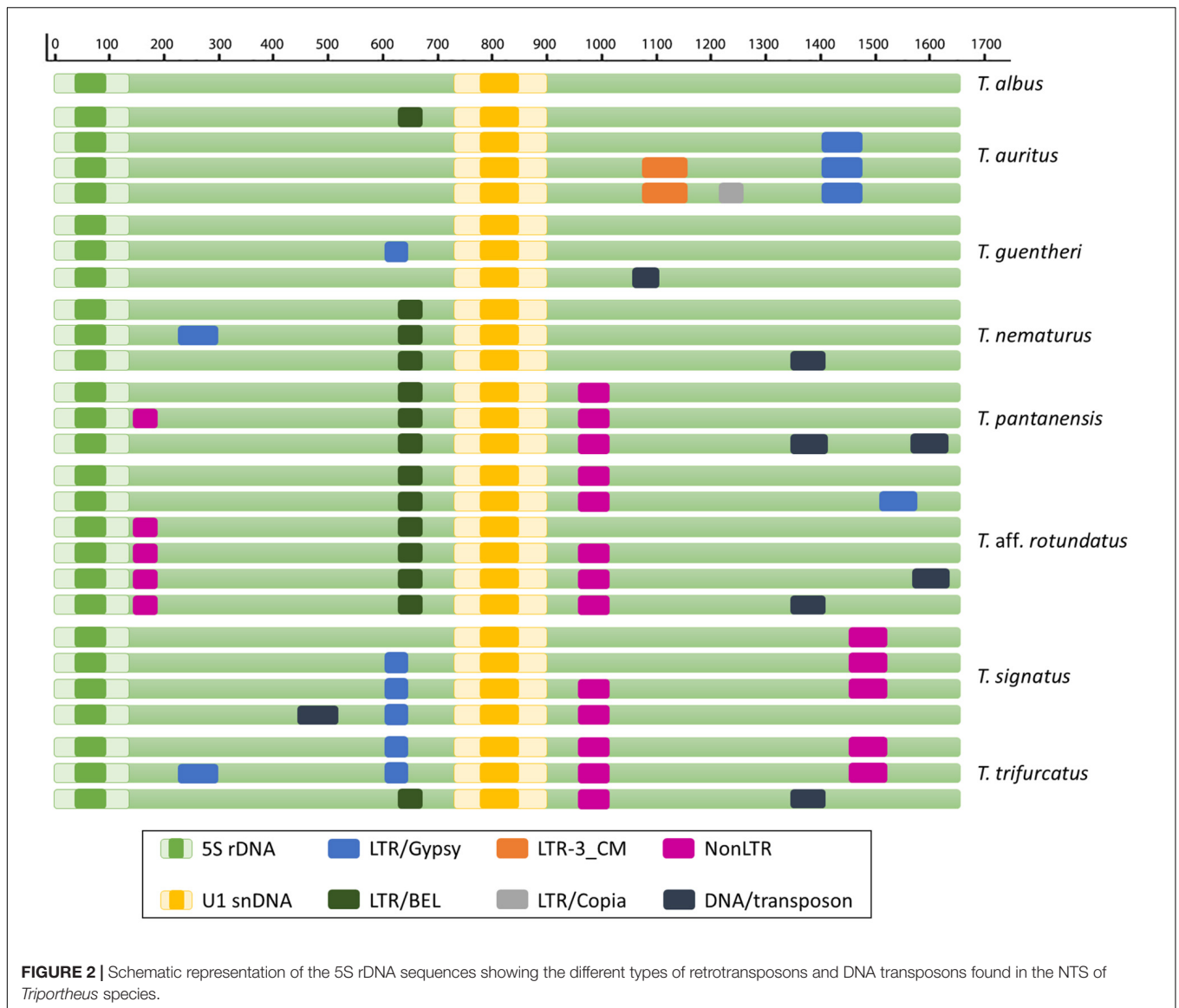
(DSE) was identified at 204 nucleotides upstream of the PSE; (iii) a 3' Box was identified at 8 nucleotides downstream of the 3' end of the gene.

The analysis using the CENSOR software identified six types of LTR Retrotransposons, six types of non-LTR retrotransposon and seven types of DNA transposon fragments in the NTS of all sequences (**Figure 2**). All species, except *T. albus*, showed fragments of several TEs; and the LTR BEL-52_DRe-I from *Danio rerio* (Howe et al., 2013) was identified in five *Tripurtheus* species.

Variability Analysis

The transcribed region of 5S rDNA showed 22 polymorphic sites (S), 23 haplotypes (h) and $0,011 \pm 0,001$ nucleotide diversity (π). As expected, the NTS displayed higher values than transcribed region, with 439 polymorphic sites, 58 haplotypes and $0,065 \pm 0,006$ of nucleotide diversity. The nucleotide variability (π) of the two transcribed regions and the two spacers within each species was low in most species, especially in the transcribed regions (**Supplementary Tables S1–S4**). *T. guentheri* showed the highest values of nucleotide variability in the transcribed regions; while *T. auritus* showed the lowest values (**Supplementary Tables S1, S3**). In the spacers, *T. trifurcatus* and *T. aff. rotundatus* presented the highest nucleotide variability (**Supplementary Tables S2, S4**).

Concerning the nucleotide divergence inferred among species (K), was low in the transcribed regions, being in many species even lower than the nucleotide variability found within each species (**Supplementary Tables S1, S3**). However, despite the



low divergence value in the transcribed regions, *T. auritus* was the species that showed the highest values of divergence and one and two fixed differences with all species in the 5S and U1 transcribed region, respectively. In addition, *T. guentheri* presented a fixed difference in the U1 transcribed region with all species, except with *T. albus*. Unlike the transcribed regions, the two spacers showed higher nucleotide divergence among species, especially *T. auritus*, since this species presented elevated values with all species analyzed (Supplementary Tables S2, S4). The number of fixed differences was much higher in the spacers, with few exceptions; *T. auritus* again presented the highest values (Supplementary Tables S2, S4).

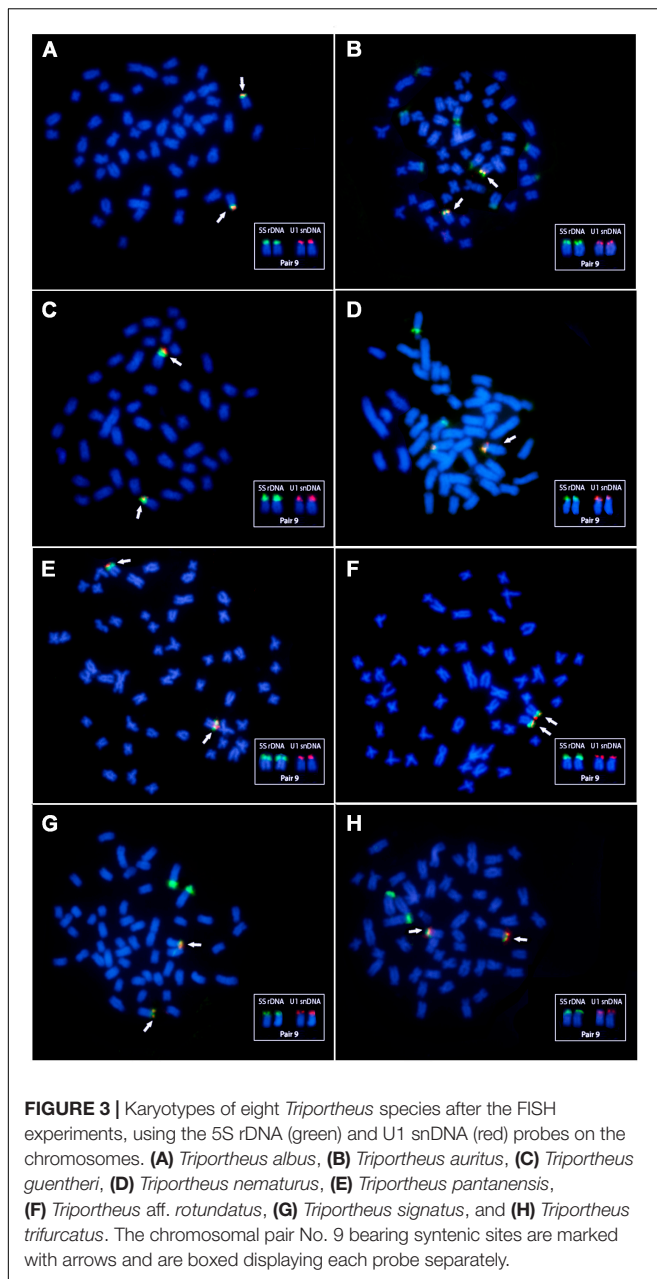
Cytogenetic Analysis

All *Triportheus* species show 52 chromosomes, and a karyotype composed by m/sm and some st chromosomes, with a heteromorphic ZZ/ZW sex chromosome system. Concerning the

double-FISH results, the 5S rDNA probe hybridized the p arms of the chromosome pair No. 9 in all species. *Triportheus albus* and *T. guentheri* showed only this site; while *T. auritus*, *T. nematurus*, *T. signatus*, and *T. trifurcatus* also showed 5S rDNA signals in the chromosome pair No. 3. *Triportheus auritus* also presented this cluster in chromosome pairs Nos. 4, 5, and 6, bearing a total of 10 sites in five chromosome pairs (Figure 3). The U1 snDNA was mapped for the first time in *Triportheus* and in each species these sequences are clustered in the chromosome pair No. 9, being co-localized with the 5S rDNA site (Figure 3).

Phylogenetic Analysis

The ML tree and the neighbor-network showed a clear clustering by species for *T. albus*, *T. auritus*, *T. guentheri*, *T. nematurus*, *T. signatus*, and *T. trifurcatus*, except for two sequences of *T. trifurcatus*. *Triportheus pantanensis* and *T. aff. rotundatus* were clustered in the same group, as two sequences of *T. trifurcatus*



(Figure 4). Both analyses showed that, although the sequences of *T. signatus* and *T. trifurcatus* are grouped by species, they are very close to each other (Figures 4, 5). The network and the ML tree also displayed that *T. auritus* is notably more distant from all species. Additionally, the ML tree evidenced that the *T. auritus* sequences occupy the most basal position in the tree (Figure 4).

DISCUSSION

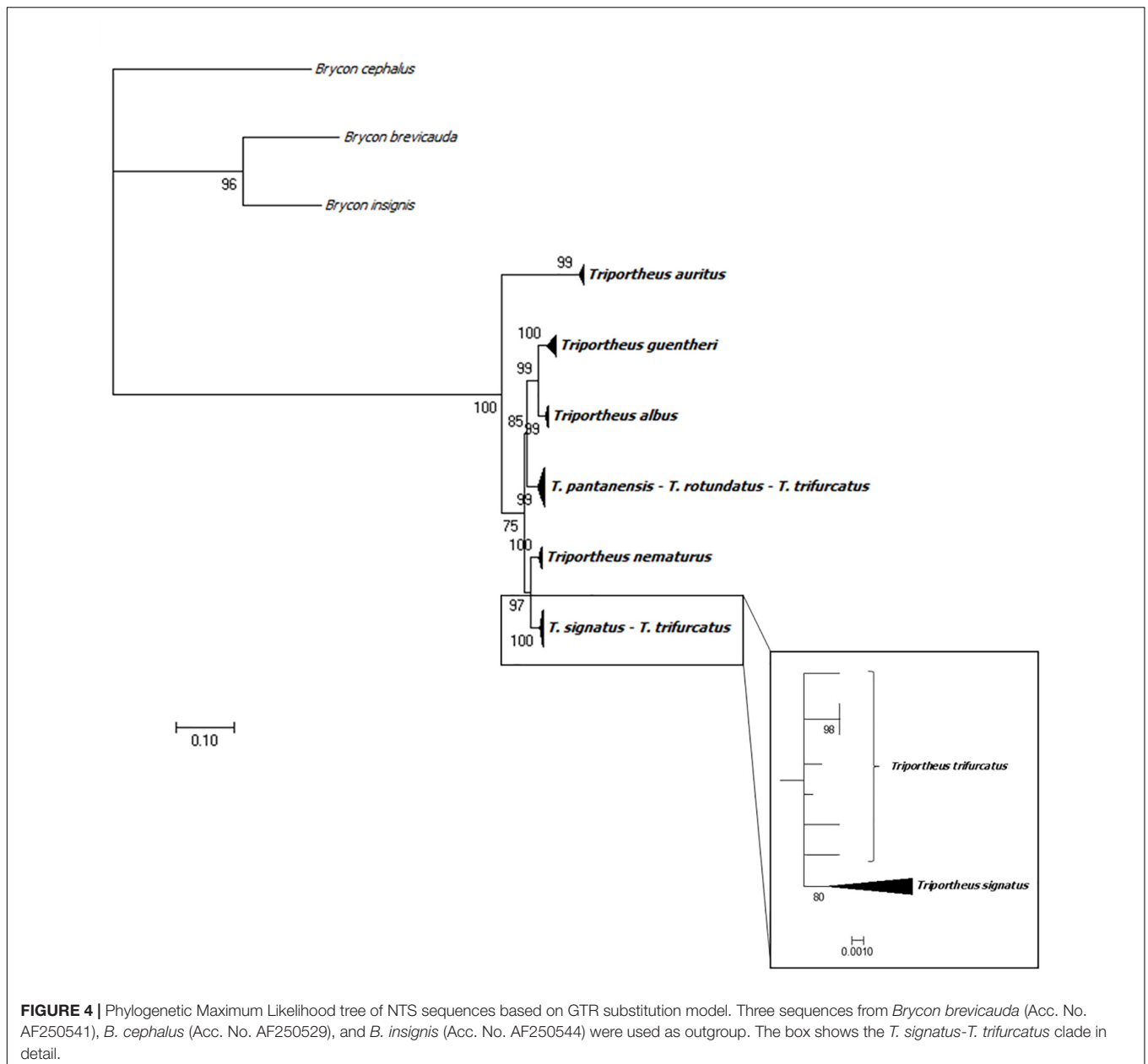
All species showed a main 5S rDNA amplicon that is presumably active, since both, ICR and the three external elements, are conserved along the 5S rDNA sequences. All these regions are

necessary for the initiation of the transcription (Cloix et al., 2000; Volkov et al., 2017). In addition, other interesting features were observed in these sequences: (i) the U1 snRNA multigene family was detected within the NTS of all *Triporthus* species by sequencing and FISH technique; (ii) higher nucleotide divergence (inside spacers) was found between *T. auritus* and all other *Triporthus* species, which is supported by previously published phylogenetic data and cytogenetic data; (iii) the presence of a varied range of TE within the spacers.

The U1 snRNA multigene family was detected within the NTS of all *Triporthus* species. This gene presented a high interspecific similarity and there were detected the regulator regions, such as the PSE, DSE and 3' box. The PSE has a conserved position at 50–60 bp upstream of the transcription starting point (McNamara-Schroeder et al., 2001) and determines the transcription start-site (Thomas et al., 1990). The DSE is an enhancer that, in humans, activates the transcription about 100-fold (Boyd et al., 2003). It is usually found about 200–250 bp upstream from PSE (Parry et al., 1989). The 3' box is a region for the transcription termination and 3' processing of the RNA (Cuello et al., 1999), and it is located about 9–19 bp downstream of the transcribed region (Barzotti et al., 2003). The conservation of these regulatory regions supports the functionality of the U1 snRNA genes.

The karyotype and 5S rDNA-FISH results agreed with previous studies (Artoni et al., 2001; Diniz et al., 2009; Marquioni et al., 2013; Yano et al., 2016, 2017b). In all species, the double-FISH results demonstrate that U1 snRNA and 5S rRNA are colocalized in the ninth chromosome pair (Figure 3), including *T. auritus*, which belongs to the first lineage that diverged from the ancestor of the genus (Mariguela et al., 2016). Such a co-localization is a plesiomorphic condition for the genus, so the analysis of 5S-U1 co-localization in other closely related genus should reinforce the taxonomic relationships of the Characiformes order. An exhaustive cytogenetic analysis using probes of ribosomal and spliceosomal RNA genes was carried out in five Characiformes species belonging to the *Astyanax* genus (Silva et al., 2015), and in four of them, 5S rDNA and U1 snDNA were localized in a same chromosome pair. These results would indicate that the linkage in a same chromosome between 5S and U1 multigene families is a common feature within the Characiformes; however, new analysis should be directed in other representative families of the order. Anyway, such a linkage is a promising marker to clarify the Characiformes taxonomy.

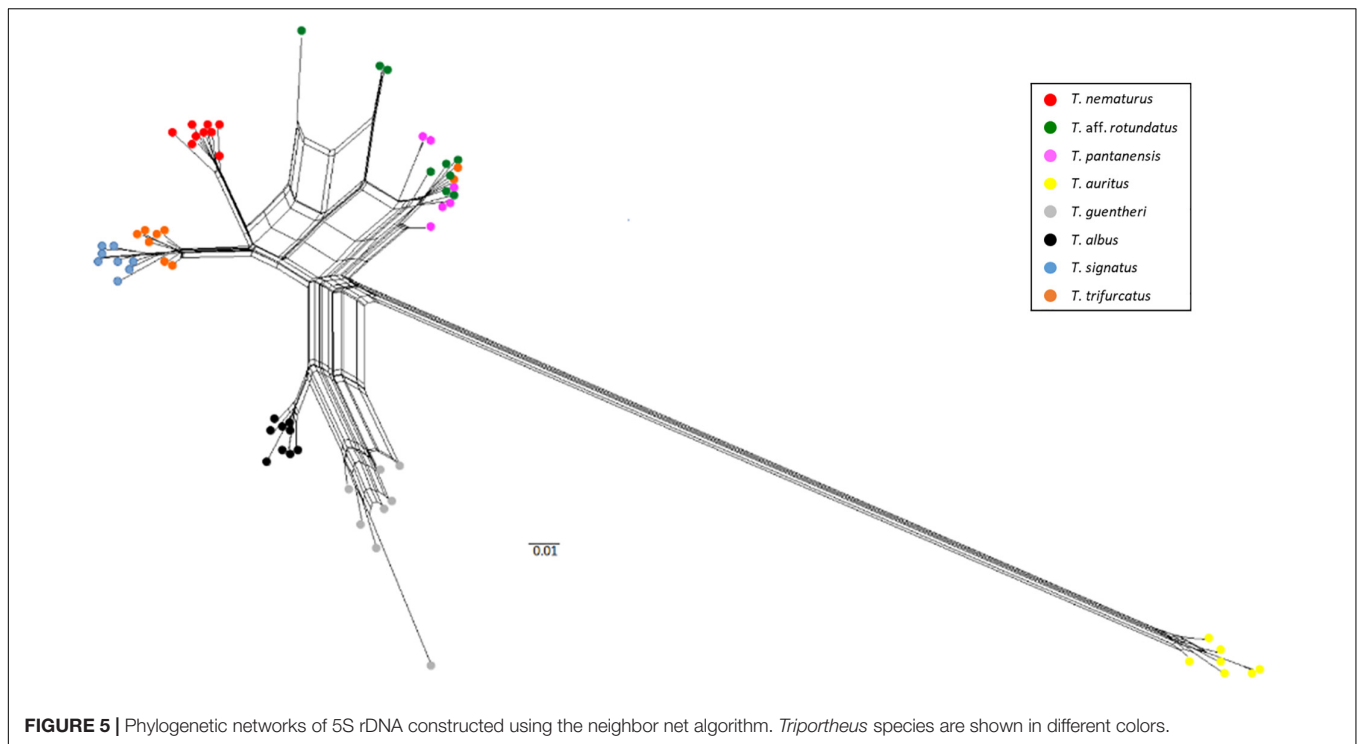
The linkage between 5S rRNA and U1 snRNA genes in only one of the chromosome pairs bearing the 5S sequences, indicate that this gene has frequently gone through mechanisms of transposition in the *Triporthus* genome, as already suggested by other authors (Drouin and Moniz de Sá, 1995). Although both genes are linked, they are transcribed by different RNA polymerases, ruling out the possibility of cotranscription (Pelliccia et al., 2001). The 5S-U1 linkage has been described in fish species such *Oreochromis niloticus* (Cabral-de-Mello et al., 2012), *Astyanax paranae*, *A. bockmanni*, *A. fasciatus* and *A. jordani* (Silva et al., 2015). A tandem linkage (co-localization) between these two multigene families has only described in the fish *A. jordani* (Silva et al., 2015) and the crustacean *Asellus aquaticus* (Pelliccia et al., 2001). *Astyanax* and *Triporthus* genus



belong to the Characoidei suborder and both species present the 5S-U1 linkage. However, in other species of the same suborder, such as *Leporinus* spp. (Ferreira et al., 2007), *Hoplias malabaricus* (Martins et al., 2006) and *Brycon* spp. (Wasko et al., 2001), no 5S-U1 tandem linkage was observed, although a U1 snRNA localization by FISH has not been tested to ascertain if the two multigene family are linked. An exhaustive analysis of such linkage in other species of the same suborder (or the Characiformes) would give new insights of the evolutionary pathway of such species.

A noteworthy characteristic of the two spacer regions is the presence of different traces of TE. The presence of TE remains has been associated with an evidence for hybridization process between species, since after hybridization occurs an explosion of

transpositions and a rapid genomic reorganization (Fontdevila, 2019). Similarly, in the species *Diplodus sargus* different TE were also described within the NTS and multiple 5S rDNA types were detected at both, electrophoretic and sequence level, probably derived from a hybridization scenario between two subspecies (Merlo et al., 2013). However, all *Triportheus* species showed one main electrophoretic band, thus indicating a higher level of intraspecific similarities than those observed in *D. sargus*. It has been postulated that *D. sargus* subspeciation resulted from a rapid series of colonization events (Summerer et al., 2001), leading to a parapatric speciation, in which the distribution boundaries could represent a hybridization area between two subspecies (Merlo et al., 2013). Commonly, the characiform fish diversification has been explained by allopatric speciation



due to river vicariance (Seehausen and Wagner, 2014), but recent works suggest that parapatric ecological speciation have important role in this group (Cooke et al., 2012; Strecker et al., 2012). Different causes could explain the differences of 5S rDNA variability between *D. sargus* and the *Triportheus* species: (i) The transposition explosion within the *Triportheus* species could be lesser than that of the *D. sargus* due to a reduced hybridization episodes among the *Triportheus* species, and (ii) the 5S-U1 linkage observed in *Triportheus* species, leads to higher selective pressure in the spacers than in the NTS of *D. sargus*, since there are more regulatory elements. Moreover, a *Tc1* retrotransposon located within NTS of the 5S rDNA has been proposed as the agent that causes the large number of 5S rDNA loci observed in *Gymnotus mamiraua* (Silva et al., 2016). Similarly, a *Rex* retroelement and a *hAT* transposon have been suggested as the causative for the 5S rDNA dispersion in *Ancistrus* and *Rinelocaria* species, respectively (Favarato et al., 2016; Glugoski et al., 2018).

Among the TE found within spacers of *Triportheus* species are several non-LTR retrotransposons. The short interspersed elements (SINEs) are a class of non-LTR retrotransposons that can be classified by the origin of the 5' region. Thus, the SINE1 type has a head derived from 7SL RNA genes (Kriegs et al., 2007), SINE2 from tRNA genes (Jurka et al., 2005) and SINE3 from 5S rRNA (Kapitonov and Jurka, 2003). Recently, a new class of SINE derived from snRNAs (U1 and U2) has been described (Kojima, 2015). Marks of non-LTR transposons have been found immediately adjacent to 5S rRNA and/or U1 snRNA genes in *T. pantanensis* and *T. aff. rotundatus*, and to U1 snRNA gene in *T. signatus* and *T. trifurcatus*. Therefore, in these species, the 5S/U1 array could act as a source of 5S and U1-derived SINEs.

Nevertheless, TE copies in the genome are sometimes non-functional due to distorted ORFs and are considered “molecular fossils” (Richardson et al., 2015).

The low values of nucleotide variability found in both, transcribed and spacer regions, demonstrate the high rate of homogenization exerted in such regions, as could be predicted by the concerted evolution model. It is well-accepted that the concerted model drives the evolution of the 5S rDNA multigene family within a given locus (Freire et al., 2010; Pinhal et al., 2011; Vizoso et al., 2011; Merlo et al., 2013). The mechanisms of unequal crossing over and gene conversion, in addition to the purifying selection in transcribed regions, act homogenizing the units of the multigene family (Nei and Rooney, 2005). In the *Triportheus* species, the repetition unit is composed by two transcribed regions and two reduced spacers with regulatory elements for two genes that are transcribed by different RNA polymerases, so the spacers also have additional selective restrictions, thus making the homogenization more effective.

On the other hand, the nucleotide divergence was high in the spacers between species, but with *T. auritus* clearly standing out, since this species showed the most elevated values when compared to other species. This high divergence between *T. auritus* and all other *Triportheus* species studied here can be perfectly associated to previous phylogenetic studies performed by Mariguela et al. (2016), since *T. auritus* was showed to be the oldest *Triportheus* species. As expected, the transcribed region showed lower divergence among species, but even in that, *T. auritus* was the species with higher divergence. Besides nucleotide divergence, *T. auritus* also shows the most divergence cytogenetic data concerning number of 5S rDNA sites (Yano et al., 2017b). While all other species show one or two chromosome

pairs bearing 5S rDNA sequences, *T. auritus* present 5 pairs (**Figure 3B**). This high number of pairs could be explained by the fact that this gene is located in the terminal regions of the chromosomes, which could favor transposition events (Nakajima et al., 2012); or by the association of 5S rDNA and transposable elements, which was clearly evidenced in this study. According to Drouin (2000), the presence of TEs can be responsible for rRNA gene movements, and this process can be displayed by the cytogenetic analysis performed here and in previous studies. Moreover, it has been stated that a double strand break within the 5S array, followed by a non-homologous recombination repair mechanism during prophase, are events that lead to share 5S sequences among chromosomes (Barros et al., 2017; Glugoski et al., 2018).

Despite of the 5S-U1 co-localization found in all *Triportheus* species, additional not U1-linked 5S loci are present in *T. signatus*, *T. nematurus*, *T. trifurcatus* and, specially, in *T. auritus*, which has four additional loci. These additional 5S rDNA arrays could be generated during the evolutionary pathway of the *Triportheus* species, helped by the TE present in the main locus. These secondary 5S arrays could not be isolated in this work, so a deep analysis of the 5S rDNA sequences should be directed. However, the presence of a possible different array of 5S rDNA could give a clue to conclude that the birth-and-death evolution model is also acting in these species, since the presence of multiple loci is a typical characteristic of this model, that arise from duplication events (Nei and Rooney, 2005). A mixed model between birth-and-death and concerted has been already described for some fish species as stingrays (Pinhal et al., 2011), *Plectorhynchus mediterraneus* (Merlo et al., 2012a), *Halobatrachus didactylus* (Merlo et al., 2012b), and *D. sargus* (Merlo et al., 2013).

The phylogenetic tree showed a robust clustering in a species-specific manner in most species. However, three out of the eight analyzed species do not grouped in a species-specific form; these are *T. pantanensis*, *T. aff. rotundatus*, and *T. trifurcatus*. This data is also supported by the highest nucleotide variability observed in *T. trifurcatus* and *T. aff. rotundatus*. Nevertheless, *T. pantanensis* presented an intermediate nucleotide variability, despite it clustered beside *T. trifurcatus* and *T. aff. rotundatus*. The species *T. pantanensis* and *T. aff. rotundatus* belong to the Paraguay and lower Paraná basins and *T. trifurcatus* to the Tocantins and Araguaia Basins, a north-south adjacent basin. Some authors postulated that the fish fauna from a specific basin could be a hybrid combination of other existing in adjacent basins (Menezes, 1988; Lima and Caires, 2011). The similarity observed in the spacer regions among these three species and the close clustering in the phylogenetic analysis, especially between *T. pantanensis* and *T. aff. rotundatus*, suggest occasional introgression process during the parapatric speciation of such species. Introgression is often an integral component of species diversification and evolution that might confer an increased genetic variation (Hedrick, 2013) or exchange beneficial alleles (Arnold and Kunte, 2017). An introgression event was described between two hake species based on 5S rDNA, microsatellites and COI markers (Miralles et al., 2014).

In this work, it has been presented for the first time an integrative genetic characterization of 5S rDNAs, using extensive analysis to show the evolutionary model of these sequences in the *Triportheus* genus. Two main facts can be extracted as conclusions: First, a tandem linkage between 5S rDNA and U1 snDNA multigene families has been found in all *Triportheus* species, including *T. auritus* (a direct representative of the first lineage that differentiated in the genus), thus indicating that this feature emerged before the genus differentiation. Secondly, many TE have been found in the spacers of the array; this fact and the close clustering of some species indicates that the evolution of the group is driven by speciation-introgression process. In conclusion, the 5S rDNA multigene family has been revealed as a good marker for the evolutionary resolution of the *Triportheus* genus, which also could be tested for others closely related Characiformes species.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The collections were authorized by the Brazilian environmental agency ICMBIO/SISBIO (License number 48628-2). The experiments followed ethical conducts, in accordance with the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (Process number CEUA 1853260315).

AUTHOR CONTRIBUTIONS

CY carried out the FISH assay. CY and SP-B carried out the cloning assay. CY, CS-J, and MM carried out the molecular and phylogenetic analysis. CY drafted the manuscript. MM helped with the discussion and drafting. LR, MC, and LB conceived and coordinated the study, participated in its design, discussed the results, and corrected the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2020.00006/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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