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Molecular phylogeny of the Neotropical fish genus *Tetragonopterus* (Teleostei: Characiformes: Characidae) ☆

Bruno F. Melo [a],*, Ricardo C. Benine [b], Gabriel S.C. Silva [a], Gleisy S. Avelino [a], Claudio Oliveira [a]

[a] *Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo, Brazil*
[b] *Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo, Brazil*

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ABSTRACT

Tetragonopterinae encompasses characid species of the genus *Tetragonopterus*, which are widely distributed throughout east of the Andes in South America. While taxonomy has recently clarified the species diversity and molecular evidence strongly supports the monophyly of *Tetragonopterus*, no interspecific relationship studies are currently available. Here we used a large molecular dataset composed of two mitochondrial and three nuclear loci containing an extensive taxon sampling within the family Characidae and included eleven species of *Tetragonopterus* to generate the first time-calibrated phylogeny for Tetragonopterinae. Our results support monophyly of the subfamily represented solely by *Tetragonopterus* and corroborate previous molecular hypothesis of close relationship with *Exodon* plus *Roeboexodon* and the subfamily Characinae. Internally, we found *Moenkhausia georgiae* as sister species to all remaining species followed by *T. rarus*, being both species endemic to the Guiana Shield drainages. Species-level relationships are first hypothesized and putative morphological apomorphies are discussed as support to monophyletic clades. Our time-calibrated phylogeny suggested an origin of the genus during the Late Oligocene–Early Miocene. We hypothesized that the Andean geological activity followed by transformations in the Amazonian hydrographic scenario during the Miocene may have promoted most of the lineage diversification within the *Tetragonopterus*.

1. Introduction

The Neotropical fish genus *Tetragonopterus* Cuvier, 1816 (Fig. 1) is widespread throughout the main South American river basins (Orinoco, Amazon, São Francisco and La Plata) and occurs additionally at the rivers systems of Guyana, Suriname, and French Guyana, and the major rivers from northeastern Brazil (Silva et al., 2013). Two synapomorphies have been proposed to define *Tetragonopterus*: the presence of only three supraneurals and a branched laterosensory canal in the sixth infraorbital (Mirande, 2010; Melo et al., 2011). In addition, several morphological features have been used to diagnose species of *Tetragonopterus*, such as the compressed and very deep body with a flattened prepelvic region, premaxillary teeth in two rows (inner row with five teeth) and a complete lateral line distinctively bent downward anteriorly (Silva et al., 2013).

Tetragonopterus was the former recipient of most small characin species that were later distributed to several new or already available genera (e.g. Eigenmann, 1903, 1908). These actions restricted *Tetragonopterus* to four species (Eigenmann, 1917) and, more recently, only to *T. argenteus* and *T. chalceus* (Reis et al., 2003). Last two decades saw an increasing number of contributions involving the alpha taxonomy and composition of the genus (Benine et al., 2004; Melo et al., 2011; Silva and Benine, 2011; Silva et al., 2013; Araujo and Lucinda, 2014) including an extensive taxonomic revision integrated with DNA barcoding (Silva et al., in preparation). Aside the well-studied alpha taxonomy of *Tetragonopterus*, the phylogenetic relationships with other genera of the Characidae remain unclear. The available morphological hypothesis suggested *Tetragonopterus* closer to Stethaprioninae, *Gymnocorymbus* and *Stichonodon* (Mirande, 2010) and, alternatively, the molecular hypotheses placed it as closer to Characinae and relatives (Javonillo et al., 2010; Oliveira et al., 2011).

Within *Tetragonopterus*, taxonomic studies associated with DNA barcoding found three well-supported mitochondrial lineages: *T. rarus*, *T. argenteus* and the large *T. chalceus* group with six species (Melo et al., 2011; Silva et al., 2013). Although some putative arrangements were discussed, these studies however did not

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* Corresponding author at: Dept. Morfologia, Instituto de Biociências, Universidade Estadual Paulista, Distr. Rubião Jr. s/n, 18618-970 Botucatu, SP, Brazil.

E-mail address: melo@ibb.unesp.br (B.F. Melo).

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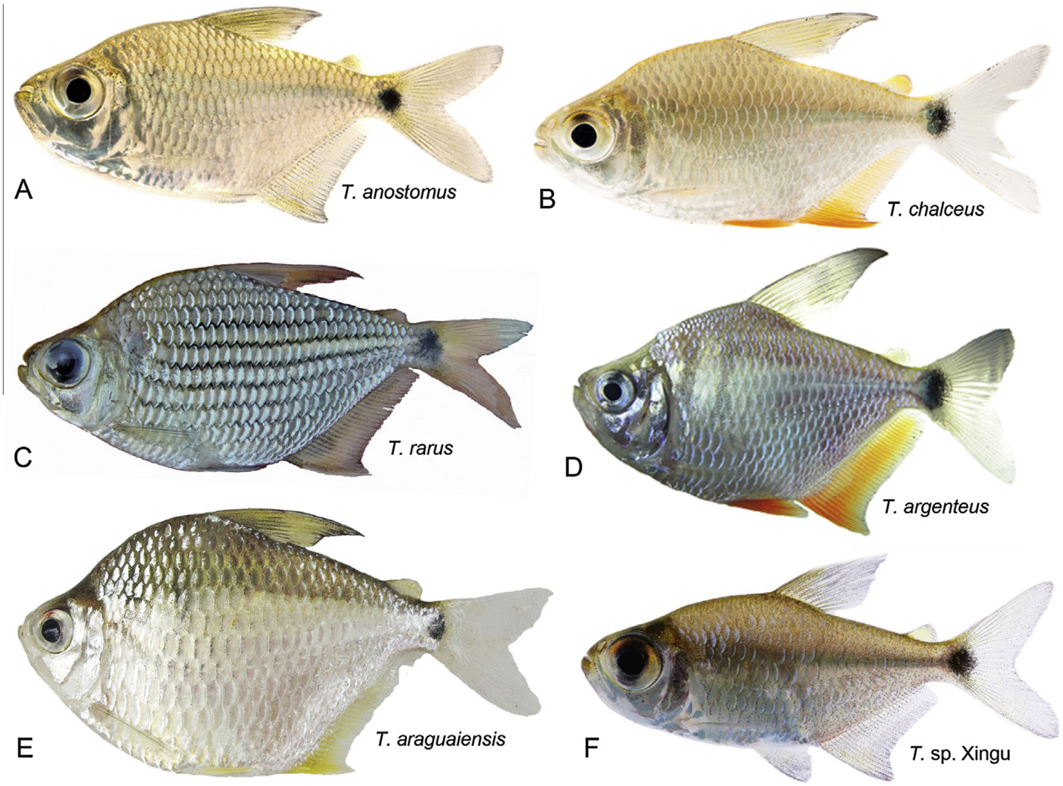

Fig. 1. Representative species of *Tetragonopterus*. (A) *T. anostomus*, Rio Tocantins, Amazon basin. (B) *T. chalceus*, Rio Xingu, Amazon basin. (C) *T. rarus*, Suriname. (D) *T. argenteus*, Río Paraguay. (E) *T. araguaiensis*, Rio Araguaia, Amazon basin. (F) *T.* sp. Xingu, Rio Xingu, Amazon basin. Photos by M.I. Taylor (A, B, F), R. Covain (C), F. Baena (D) and B.F. Melo (E).

reconstruct formal phylogenetic relationships due the limited coverage of their dataset composed by a single mitochondrial locus commonly used for species discrimination (Hebert et al., 2003). As such, neither morphological nor molecular-based phylogenetic hypothesis among species of *Tetragonopterus* currently exist.

To resolve these apparent conflicts regarding genus-level relationships between *Tetragonopterus* and other characids, and also to reconstruct the unknown species-level phylogeny for the genus, we undertook a new molecular-based analysis containing all recognized species of *Tetragonopterus*. We incorporated these species into a large molecular dataset of the Characidae (Oliveira et al., 2011), generated a time-calibrated phylogeny using the available fossil record and provided a well-supported framework for future evolutionary studies within the genus.

2. Materials and methods

2.1. Taxon sampling and morphological comparisons

Voucher specimens were fixed in 95% ethanol or 10% formalin and then transferred to 70% ethanol for permanent storage. Ingroup taxa included 28 specimens covering a total of seven described species of *Tetragonopterus* plus three other putative new species and *Moenkhausia georgiae* (Table 1). Outgroup contains 192 characiform taxa with a species-rich coverage of the Characidae previously generated using the same loci (Oliveira et al., 2011). Comparative morphological analyses were conducted in the following taxa: *Charax leticiae*: LBP 7600, 1, 59.0 mm Standard Length (SL); *Exodon paradoxus*: LBP 8851, 47.1–58.3 mm SL; *Gymnocorymbus ternetzi:* LBP 8443, 13, 21.2–45.3 mm SL; *Moenkhausia georgiae*: ANSP 94708, 1 paratype, 57.9 mm SL; *M. xinguensis*: MZUSP 36806, 1, 46.8 mm SL; *Poptella compressa*: MCP 25629, 2, 43.6–46.1 mm SL; *P. paraguayensis*: MZUSP 36715, 1 cleared and stained (c&s), 55.9 mm SL; LIRP 4565, 1 c&s, 54.6 mm SL; *Roeboexodon* sp.: LBP 16101, 1, 82.8 mm SL; *Roeboides bonariensis*: LBP 9248, 2, 75.5–82.4 mm SL; *Stichonodon insignis*: LIRP 5083, 2 c&s, 35.0–37.5 mm SL; *Tetragonopterus anostomus*: LBP 7687, 17 paratypes, 34.6–38.9 mm SL; *T. araguaiensis*: MZUSP 111003, 5 paratypes, 48.4–53.0 mm SL; *T. argenteus*: MZUSP 15570, 2 c&s, 58.9–67.3 mm SL; *T. carvalhoi*: LBP 5376, 30 paratypes, 29.7–45.8 mm SL; *T. chalceus*: MZUSP 29817, 2, 44.9–47.1 mm SL; *T. denticulatus*: LBP 1585, 6 paratypes (one c&s), 48.5–61.7 mm SL; *T. rarus*: LBP 5375, 1, 38.0 mm SL; *T.* sp. São Francisco: LBP 10294, 21, 70.7–91.3 mm SL; *T.* sp. Xingu: MZUSP 91950, 60, 52.6–39.1 mm SL; *T.* sp. Tapajós: LBP 13949, 3, 62.8–65.9 mm SL. Osteological analysis was conducted on cleared and counterstained specimens following methods of Taylor and Van Dyke (1985) and Weitzman (1974). Museum abbreviatures follow Sabaj Pérez (2013).

2.2. DNA extraction and sequencing

Genomic DNA was extracted from tissue muscles using a DNeasy Tissue kit (Qiagen Inc.; www.qiagen.com) according to manufacturer's instructions. We used one round of polymerase chain reaction (PCR) to amplify three mitochondrial loci, the *16S*, *Cytochrome b* (Cytb) and *Cytochrome oxidase c subunit 1* (Coi). In addition, we obtained sequences of the nuclear *myosin heavy chain 6* gene (*Myh6*), *recombination activating gene 1* (*Rag1*), and *recombination activating gene 2* (*Rag2*) following the nested-PCR procedures obtained in Oliveira et al. (2011). We used 12.5 µl as a total volume containing 9.075 µl of double-distilled water, 1.25 µl 5× reaction buffer, 0.375 µl $MgCl_2$, 0.25 µl dNTP mix at 8 mM, 0.25 µl of each primer at 10 µM, 0.05 µl Platinum Taq

Table 1
Taxa, vouchers and locality information of the analyzed specimens of _Tetragonopterus_. Asterisks after species names represent analyzed paratypes.

Taxon	Voucher	Specimen	Locality	Geographic coordinates	City, state	Country
_Tetragonopterus anostomus_*	LBP 7687	43527	Rio Araguaia, Amazon basin	−14.37666667 −50.66888889	Cocalinho, Mato Grosso	Brazil
_Tetragonopterus anostomus_*	LBP 7687	43528	Rio Araguaia, Amazon basin	−14.37666667 −50.66888889	Cocalinho, Mato Grosso	Brazil
_Tetragonopterus araguaiensis_*	LBP 4154	11693	Rio Araguaia, Amazon basin	−15.89311111 −52.25025000	Aragarças, Goiás	Brazil
_Tetragonopterus araguaiensis_*	LBP 7756	36372	Rio Araguaia, Amazon basin	−13.32300000 −50.62241667	Cocalinho, Mato Grosso	Brazil
Tetragonopterus argenteus	LBP 3059	19144	Río Orinoco, Orinoco basin	7.63655556 −66.31783333	Caicara del Orinoco, Bolívar	Venezuela
Tetragonopterus argenteus	LBP 3758	22029	Rio Negro, Paraguay basin	−19.57602778 −56.24708333	Aquidauana, Mato Grosso do Sul	Brazil
Tetragonopterus argenteus	LBP 5535	27201	Rio Balsas, Parnaíba basin	−7.54055556 −46.03916667	Balsas, Maranhão	Brazil
Tetragonopterus argenteus	LBP 12578	54261	Río Marañon, Amazon basin	−4.13711111 −73.52113889	Nauta, Loreto	Peru
_Tetragonopterus carvalhoi_*	LBP 5306	26906	Rio Jari, Amazon basin	−0.56750000 −52.57805556	Laranjal do Jari, Amapá	Brazil
_Tetragonopterus carvalhoi_*	LBP 5306	26907	Rio Jari, Amazon basin	−0.56750000 −52.57805556	Laranjal do Jari, Amapá	Brazil
Tetragonopterus chalceus	LBP 12581	54266	Río Marañon, Amazon basin	−4.30377777 −73.5211388	Nauta, Loreto	Peru
Tetragonopterus chalceus	LBP 8773	44050	Rio Araguaia, Amazon basin	−13.3230000 −50.62241667	Cocalinho, Mato Grosso	Brazil
Tetragonopterus chalceus	LBP 8857	44293	Rio Araguaia, Amazon basin	−13.37669444 −50.66900000	Cocalinho, Mato Grosso	Brazil
Tetragonopterus chalceus	LBP 13946	56694	Rio Tapajós, Amazon basin	−4.28041667 −59.99058333	Itaituba, Pará	Brazil
Tetragonopterus chalceus	LBP 16911	64564	Rio Xingu, Amazon basin	−13.15377778 −51,92186111	Ribeirão Cascalheira, Mato Grosso	Brazil
_Tetragonopterus denticulatus_*	LBP 1629	11694	Rio Araguaia, Amazon basin	−15.89311111 −52.25025000	Aragarças, Goiás	Brazil
_Tetragonopterus denticulatus_*	LBP 5751	28113	Rio Araguaia, Amazon basin	−15.89208333 −52.25055556	Aragarças, Goiás	Brazil
Tetragonopterus rarus	LBP 5375	27017	Rio Jari, Amazon basin	−0.56416667 −52.57916667	Laranjal do Jari, Amapá	Brazil
Tetragonopterus rarus	MHNG 2718.028	SU08-640 (172-21)	Paloemeu Rivier, Marowijne basin	3.17833 −55.4192	Sipaliwini	Suriname
Tetragonopterus rarus	MHNG 2735.090	SUGF12-362 (172-27)	Kabalebo Rivier, Corantijn basin	4.45058 −57.1631	Sipaliwini	Suriname
Tetragonopterus sp. São Francisco	LBP 8268	37556	Rio São Francisco	−15.32338888 −43.66458333	Jaíba, Minas Gerais	Brazil
Tetragonopterus sp. São Francisco	LBP 10394	48897	Rio São Francisco	−17.32566667 −44.76694444	Buritizeiro, Minas Gerais	Brazil
Tetragonopterus sp. Tapajós	LBP 13949	56698	Rio Tapajós, Amazon basin	−4.45819444 −56.26750000	Itaituba, Pará	Brazil
Tetragonopterus sp. Tapajós	LBP 13949	56699	Rio Tapajós, Amazon basin	−4.45819444 −56.26750000	Itaituba, Pará	Brazil
Tetragonopterus sp. Xingu	LBP 15711	64561	Rio Xingu, Amazon basin	−13.15377778 −51.92186111	Ribeirão Cascalheira, Mato Grosso	Brazil
Tetragonopterus sp. Xingu	LBP 15711	64563	Rio Xingu, Amazon basin	−13.15377778 −51.92186111	Ribeirão Cascalheira, Mato Grosso	Brazil
Moenkhausia georgiae	MHNG 2716.097	SU08-848 (172-22)	Tapanahony Rivier, Marowijne basin	3.366 −55.4321	Sipaliwini	Suriname
Moenkhausia georgiae	MHNG 2716.097	SU08-849 (172-23)	Tapanahony Rivier, Marowijne basin	3.366 −55.4321	Sipaliwini	Suriname

DNA polymerase enzyme (Invitrogen; www.invitrogen.com) and 1.0 μl genomic DNA (10–50 ng). Primers were obtained from the literature: 16s (Palumbi, 1996); Cytb, Rag1 and Rag2, 1st PCR (Oliveira et al., 2011); Coi (Melo et al., 2011); Myh6 (Li et al., 2007); Rag2, 2nd PCR (Lovejoy and Collette, 2001). The PCR consisted of an initial denaturation (4 min at 95 °C) followed by 28–30 cycles of chain denaturation (30 s at 95 °C), primer hybridization (30–60 s at 48–54 °C), and nucleotide extension (30–60 s at 72 °C). After visualization of the fragments using 1% agarose gel, we performed the sequencing reaction using dye terminators (BigDye™ Terminator v 3.1 Cycle Sequencing Ready Reaction Kit, Applied Biosystems; www.appliedbiosystems.com) purified again through ethanol precipitation. Samples were then loaded onto an automatic sequencer ABI 3130-Genetic Analyzer (Applied Biosystems) at the Universidade Estadual Paulista, Botucatu, São Paulo, Brazil.

2.3. Alignment and phylogenetic analyses

We assembled and edited consensus sequences in Geneious 7.1.7 (www.geneious.com; Kearse et al., 2012) applying IUPAC ambiguity codes where we detected uncertainty of nucleotide identity. We then aligned the consensus sequences of each gene using Muscle algorithm (Edgar, 2004) and inspected alignments by eye for major misalignments and concatenated these into a reduced Characidae matrix containing five loci (Oliveira et al., 2011). In order to reduce computation time and to resolve internal nodes, we used a reduced dataset (six loci) containing only species of _Tetragonopterus_ and _Moenkhausia georgiae_, along with _Exodon_ and _Roeboexodon_, Characinae, Iguanodectidae, Acestrorhynchidae and Triportheidae. To evaluate the occurrence of substitution saturation, we estimated the index of substitution saturation in asymmetrical (Iss.cAsym) and symmetrical (Iss.cSym) topologies using

Dambe 5.3.38 (Xia, 2013). Maximum likelihood (ML) trees for each locus were generated in order to test for incongruence between different loci and identify potentially contaminated sequences. A set of six reasonable partitioning schemes, ranging from 1 to 13 partitions, was tested following the procedures outlined by Li et al. (2008) using the Akaike information criterion (AIC) and Bayesian information criterion (BIC) (results not shown). The best-fit model of nucleotide substitution was searched in Mega 5.04 (Tamura et al., 2011) under default parameters using the AIC (see Posada and Buckley, 2004 for justification).

A maximum likelihood (ML) analysis of the partitioned data (13 partitions) was performed in RAxML HPC2 on XSEDE (Stamatakis, 2006) implemented on CIPRES (Miller et al., 2010). We used random starting trees with GTR+G model (Stamatakis et al., 2008), and keep other parameters at default values. All ML analyses were conducted following the 13 partitions scheme as suggested by the AIC and BIC. Topological robustness was investigated using 1000 non-parametric bootstrap replicates.

2.4. Divergence time and node-age estimations

The estimation of divergence time in the ML phylogeny was carried out using Beast 1.8.0 (Drummond et al., 2012) on a reduced dataset that included the subfamily Tetragonopterinae along with members of Characinae, *Exodon*/*Roeboexodon*, and representatives of the families Acestrorhynchidae, Iguanodectidae and Triportheidae. To calibrate our molecular tree we followed the guideline proposed by Parham et al. (2012). †*Lignobrycon ligniticus* (Woodward, 1898) (type specimen: BMNH P9012) is sister to *L. myersi* and this clade is sister to *Triportheus* (Malabarba, 1998) within the Triportheidae. Both *L. myersi* and *Triportheus* were included in our dataset.

†*Lignobrycon ligniticus* was described based on complete specimens collected in the Tremembé Formation, Taubaté Basin, São Paulo, Brazil. A geological study (Riccomini et al., 1991) dated the formation for the Oligocene, as well as suggested by paleontological studies with mammals (Soria and Alvarenga, 1989) and pollens (Lima et al., 1985). Following the International Commission on Stratigraphy (www.stratigraphy.org), the Oligocene extended from 33.9 to 23.03 million of years ago (Ma). These dates were implemented in BEAST with a log-normal prior offset with a mean and standard deviation of 28.5 ± 5.5. We used a birth–death model for the speciation likelihood and a random starting tree. The analysis was performed with 10 million generations with sampled trees every 1000th generation. Stationarity and sufficient mixing of parameters (ESS > 200) were checked using Tracer 1.5 (Rambaut et al., 2014). A final tree was built using FigTree v.1.4.2.

3. Results

The concatenated matrix included 4663 bp spanning 220 species of the Characidae (*sensu* Oliveira et al., 2011) and other six families of the Characiformes. The reduced dataset contains 5047 bp of 48 species, including species of *Tetragonopterus* and *Moenkhausia georgiae*, along with *Exodon* and *Roeboexodon*, Characinae, Iguanodectidae, Acestrorhynchidae and Triportheidae. Content information and characteristics of each molecular data partition are represented in Table 2. All data were further tested to investigate the occurrence of substitution saturation and the results did not show any significant saturation. All generated sequences are deposited on GenBank with accession numbers: 16S KT880470–KT880492, Cytb KT895104–KT895123, Myh6 KT895124–KT895143, Rag1 KT895144–KT895163, Rag2 KT895164–185, and Coi KT895186–KT895198.

Our molecular phylogeny (Fig. 2) strongly supports a monophyletic Tetragonopterinae that contains only species of *Tetragonopterus* plus *Moenkhausia georgiae* as sister to *Exodon* and

Table 2
Content information and characteristics of each molecular data partition. Upper line represents values for Matrix 1 (total dataset) and lower line represents values for Matrix 2 (Tetragonopterinae and allied species).

	16S	CytB	Myh6	Rag1	Rag2	Coi	Total
Number of sequences	220 (100%) 44 (91.7%)	202 (91.8%) 40 (83.3%)	203 (92.2%) 37 (77.1%)	202 (91.8%) 37 (77.1%)	200 (90.9%) 41 (85.4%)	– 39 (81.3%)	220 48
Base pairs after alignment	620 606	992 992	755 741	1265 1028	1031 1023	– 657	4663 5047
Number of variable sites	249 220	652 492	352 194	760 347	918 367	– 276	3031 1896
Number of informative characters under parsimony	294 172	542 428	295 139	596 216	539 243	– 241	2266 1439
% informative characters under parsimony	47.4 28.4	54.6 43.1	39.1 18.7	47.1 21.0	52.3 23.8	0 36.7	48.6 28.5
Π_T	0.232 0.231	0.304 0.299	0.245 0.248	0.224 0.222	0.230 0.234	– 0.311	0.246 0.255
Π_C	0.231 0.227	0.283 0.290	0.215 0.213	0.241 0.227	0.254 0.250	– 0.261	0.247 0.246
Π_A	0.315 0.318	0.266 0.268	0.305 0.306	0.250 0.252	0.243 0.242	– 0.251	0.270 0.269
Π_G	0.222 0.224	0.246 0.143	0.235 0.234	0.285 0.299	0.274 0.274	– 0.178	0.237 0.230
Overall mean genetic distance (*p*-distance)	0.119 ± 0.007 0.088 ± 0.006	0.197 ± 0.007 0.185 ± 0.008	0.066 ± 0.004 0.050 ± 0.004	0.089 ± 0.004 0.061 ± 0.004	0.088 ± 0.004 0.062 ± 0.004	– 0.161 ± 0.010	0.104 ± 0.002 0.112 ± 0.003
Nucleotide substitution model	GTR+I+Γ GTR+I+Γ	GTR+I+Γ HKY+I+Γ	T92+I+Γ T92+Γ	K2P+I+Γ K2P+Γ	K2P+Γ K2P+Γ	– GTR+I+Γ	GTR+I+Γ GTR+I+Γ
α (shape) parameter of Γ distribution	0.63 0.41	0.63 0.91	1.03 –	0.77 0.33	0.43 0.39	– 0.93	0.57 0.46
Proportion of invariants (I) sites	0.35 0.39	0.35 0.44	0.46 –	0.30 –	– –	– 0.53	0.40 0.39

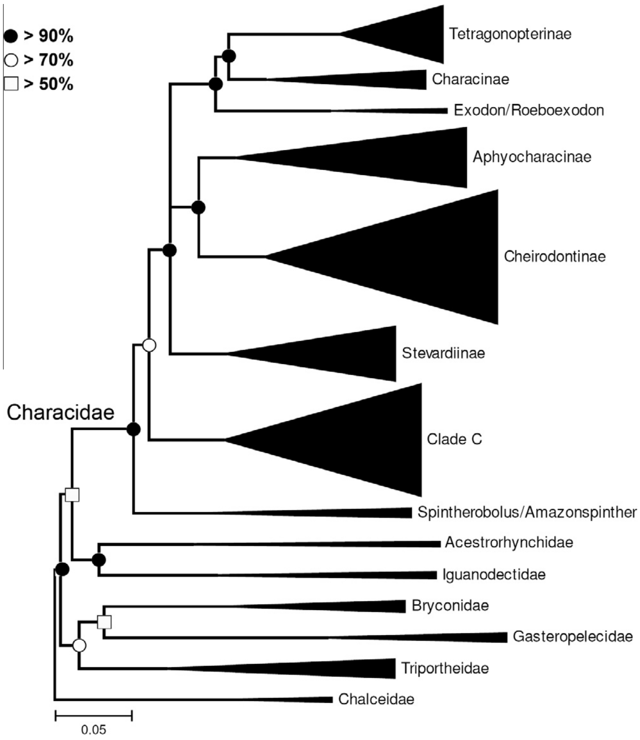

Fig. 2. Summary of the relationships among major lineages of Characidae and related families obtained by a maximum likelihood analysis of the concatenated dataset.

Roeboexodon. This clade appeared sister to the subfamily Characinae. Evidence for generic allocation of *Moenkhausia georgiae* within *Tetragonopterus* are being provided by Silva et al. (in preparation). The best-supported time-calibrated phylogeny (Fig. 3) estimated a Late Oligocene crown age for the clade composed by Characinae, *Exodon*/*Roeboexodon* and Tetragonopterinae (25 Ma; 95% highest posterior density, HPD, 15–36 Ma) and a Late Oligocene–Early Miocene crown age for *Tetragonopterus* and the clade composed by *Exodon* and *Roeboexodon* (23 Ma, 14–33 Ma HPD).

The first two internal ramifications within the Tetragonopterinae occurred contemporaneously during the middle Miocene (Fig. 3). First, *Moenkhausia georgiae* split from the remaining *Tetragonopterus* approximately 18 Ma (10–26 Ma HPD), and *T. rarus* split from the remaining congeners at around 15 Ma (9–22 Ma HPD). The Miocene was also the time for diversification of several subclades within the *Tetragonopterus* as, for example, the split between the clade of *T. anostomus*, *T. denticulatus*, and *Tetragonopterus* sp. Xingu and the remaining *Tetragonopterus* that occurred ~13 Ma (7–18 Ma HPD). Radiations within the clade (*T. denticulatus* (*T. anostomus* + *T.* sp. Xingu)) began at approximately 7 Ma (3.5–10.5 Ma HPD). Late Miocene was the time that *Tetragonopterus argenteus* split from the remaining *Tetragonopterus* at ~10 Ma (5.7–14.9 Ma HPD), and *T. chalceus* separated from the less inclusive clade at 8.3 Ma (4.6–12 Ma HPD). We also estimated that the clade ((*T. carvalhoi* + *T.* sp. São Francisco) (*T. araguaiensis* + *T.* sp. Tapajós)) split from one another ~7 Ma (4–10 Ma HPD) and those internal radiations dated to Late Miocene–Early Pliocene (5 Ma, 2–9 Ma HPD).

4. Discussion

4.1. Phylogenetic relationships within the Tetragonopterinae

Tetragonopterinae, known as American tetras, was recognized for a long time as a large fish assemblage containing "the most successful characoids" of the neotropics (Géry, 1977). However, the lack of synapomorphies supporting such a large clade led Reis (2003) to restrict the subfamily to *Tetragonopterus* and the remaining 87 genera were allocated as *incertae sedis* in Characidae (Lima et al., 2003). Tetragonopterinae was subsequently re-expanded to include *Tetragonopterus* and 18 more characid genera in a morphological analysis of the Characidae (Mirande, 2010). The proposed synapomorphy that supports the subfamily is the presence of two uroneurals, a character state that occurs homoplastically across the Characidae (Mirande, 2010). Subsequent molecular phylogenies (Javonillo et al., 2010; Oliveira et al., 2011) and a morphological phylogeny of the Characinae (Mattox and Toledo-Piza, 2012) did not corroborate such morphological arrangement. Mirande (2010) also hypothesized *Tetragonopterus* as the sister genus of the clade containing *Gymnocorymbus*, *Stichonodon*, and Stethaprioninae (*Poptella* and *Stethaprion*) supported by four non-exclusive synapomorphies. Again, this placement was not corroborated by molecular studies (e.g. Oliveira et al., 2011).

Molecular phylogenies alternatively concord in recognizing the analyzed species of *Tetragonopterus* as sister to Characinae (Javonillo et al., 2010) including *Microschemobrycon*, and with *Exodon* and *Roeboexodon* (Oliveira et al., 2011). Interestingly, Mattox and Toledo-Piza (2012) used morphological data to redefine Characinae with the resolution (Phenacogasterini (Characini + Cynopotamini) and found *Tetragonopterus* as its closest sister group. Additional molecular studies focusing on the Aphyocharacinae, Cheirodontinae, Stevardiinae and *Gymnocorymbus* added more taxa and corroborated *Tetragonopterus* closer to Characinae (Tagliacollo et al., 2012; Mariguela et al., 2013; Thomaz et al., 2015; Benine et al., 2015). Here, we found a well-supported monophyletic clade of Tetragonopterinae (including all species of *Tetragonopterus* plus *Moenkhausia georgiae*) sister to *Exodon* plus *Roeboexodon* and this clade sister to Characinae (including *Microschemobrycon*) (Figs. 2 and 3), which agrees with both molecular and morphological hypotheses (Oliveira et al., 2011; Mattox and Toledo-Piza, 2012) with the sole exception of Mirande (2010).

Prior taxonomic studies using both morphology and DNA barcoding revealed the presence of three mitochondrial lineages within *Tetragonopterus* (Melo et al., 2011; Silva et al., 2013). Our multilocus analyses with all recognized species of *Tetragonopterus* found a higher number of monophyletic, strongly supported clades. The first clade to diversify is *Moenkhausia georgiae* from the Guianas, a species formally assigned as a member of *Moenkhausia* (Géry, 1965). Generic allocation of *M. georgiae* is perhaps unsurprising due the overall non-homologous similarities with some deep-bodied species of *Moenkhausia* (e.g. *M. chrysargyrea*, *M. grandisquamis*). However, a taxonomic review of *Tetragonopterus* (Silva et al., in preparation) provides evidence for a new allocation of the species within *Tetragonopterus*. The sister clade to *M. georgiae* is *T. rarus* (a species from the Guianas and Rio Jari of the lower Amazon basin) as the sister group of the remaining species. Silva et al. (2013) found that *T. rarus* represents a distinct mitochondrial lineage within *Tetragonopterus*, a result consistent with our multilocus phylogeny.

The sister clade to *Tetragonopterus rarus* contains nine species (*T. anostomus*, *T. araguaiensis*, *T. argenteus*, *T. carvalhoi*, *T. chalceus*, *T. denticulatus*, *T.* sp. São Francisco, *T.* sp. Tapajós, and *T.* sp. Xingu). These share the presence of a pronounced downward curvature of the anterior portion of the pored scales, a feature not observed neither in *Moenkhausia georgiae* nor *T. rarus*. Such novelty is likely due the modification in the entire structure and disposition of the horizontal scale rows along the body carrying out the laterosensory canal in the same way down. Such derived condition of the downward curvature of the lateral line may be interpreted, in light of our phylogeny, as a putative synapomorphy for this major, less inclusive clade of *Tetragonopterus*. Further comparative studies focusing

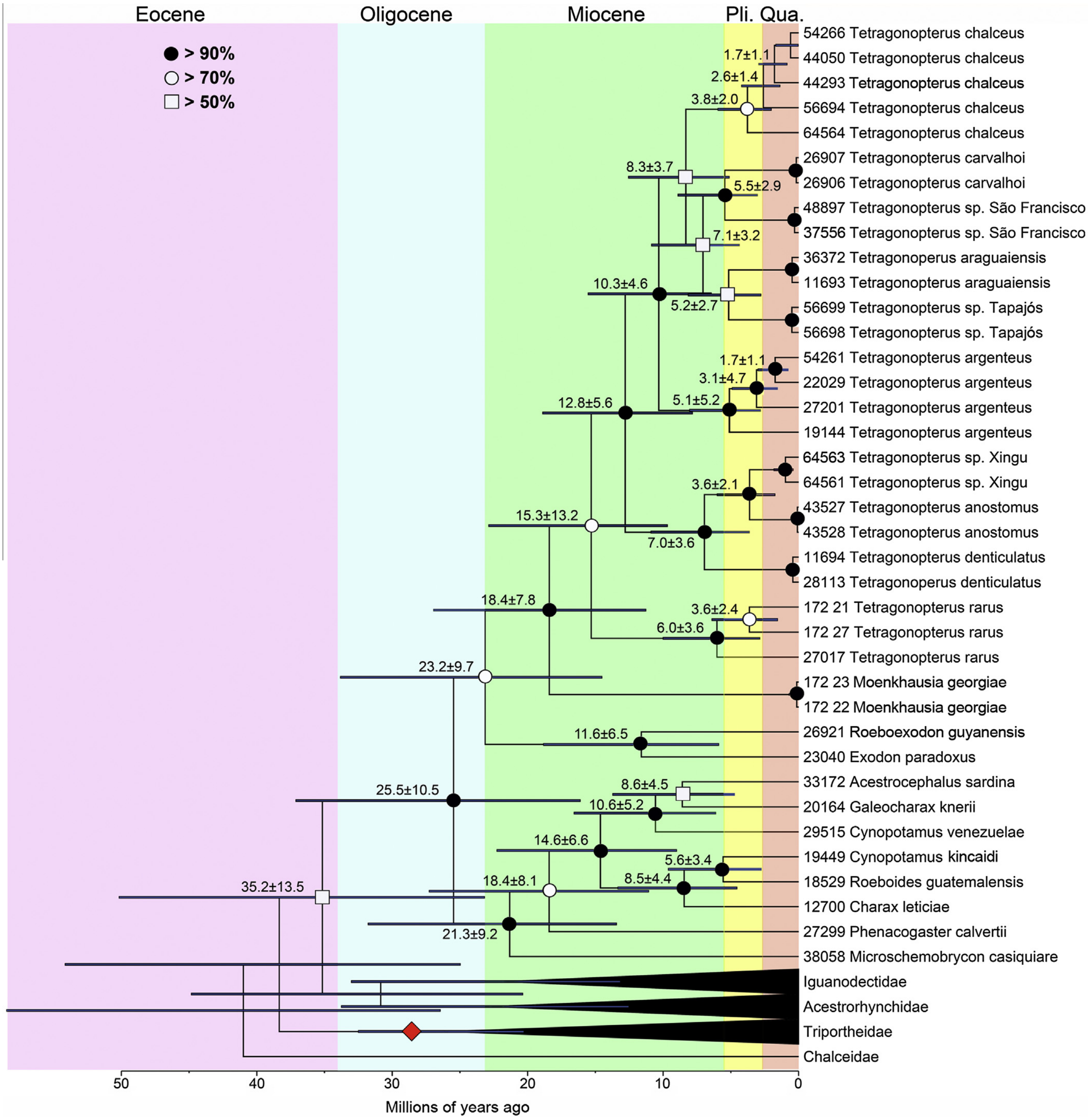

Fig. 3. Timetree of Tetragonopterinae and relatives obtained from maximum likelihood relaxed clock analysis of the reduced dataset using †*Lignobrycon ligniticus* (Triportheidae) as calibration point (red diamond). Horizontal node bars indicate dating estimations with 95% posterior probability densities (HPD). Numbers before species names represent voucher information. Pli. = Pliocene; Qua. = Quaternary. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

on how these modifications occur ontogenetically across the Tetragonopterinae may address important information on the character evolution.

Tetragonopterus denticulatus is sister to *T. anostomus* and *T.* sp. Xingu, all three species from the Brazilian Shield in the Araguaia and Xingu river systems of the eastern Amazon basin. These three species share the presence of numerous (never less than five) and minuscule teeth in the lower jaw that may represent a putative synapomorphy to be confirmed in a more comprehensive morphological analysis. Internal diversification within this three-species clade involved, among others, modifications in dentition, mouth morphology and pigmentation (Silva et al., in preparation). As example, the presence of only one conspicuous humeral mark unites the species pair *T. anostomus* and *T.* sp. Xingu.

Tetragonopterus argenteus is well supported as a monophyletic species and appeared sister to the remaining species. Melo et al. (2011) proposed *T. argenteus* as a species complex with multiple, biogeographically structured mitochondrial lineages. We accordingly found that subsequent multiple radiations occurred during the Plio-Pleistocene (~5–2 Ma) within the *T. argenteus*. The specimen from the Orinoco basin in Venezuela, for example, split from the other three specimens at around 5 Ma and subsequent radiations occurring at ~3–2 Ma that split populations from the Rio Parnaíba of the Northeastern Brazil and then the sister pair composed by specimens from the Río Marañon and Rio Paraguay. Such strong evidence provides foundation to corroborate Melo et al.'s (2011) hypothesis of the presence of sibling species within the present concept of *T. argenteus*. The presence of more than 10 scales in the predorsal line, from the tip of the occipital spine to the origin of dorsal fin, may represent a putative synapomorphy and, thus, a definition for the herein called *T. argenteus* species complex.

Our phylogeny supports monophyly for the five analyzed specimens of *Tetragonopterus chalceus*, the most widespread species of the genus together with *T. argenteus*. The taxonomic revision shows that populations from distinct drainages are morphologically undistinguishable from each other and that only one name should be currently used to address this form, the *T. chalceus* (Silva et al., in preparation). The taxonomy of both *T. argenteus* and *T. chalceus* would better benefit from further investigation through alternative techniques (e.g. geometric morphometrics) and/or population genetics with extensive sampling to better determine the limits of this putative species. Indeed, adequate sample sizes relative to South American fishes are still a bottleneck to such comparative studies and, as a consequence, a long way likely exists to have such hypothesis tested.

Tetragonopterus carvalhoi is highly supported as sister to *T.* sp. São Francisco and *T. araguaiensis* sister to *T.* sp. Tapajós with moderate support. The modified lozenge-shaped mark on the caudal peduncle that diagnoses *T. carvalhoi* (Melo et al., 2011) is unique among *Tetragonopterus* species and may represent an autapomorphic condition to be further tested in more inclusive morphological studies. Specimens of *Tetragonopterus* sp. São Francisco share the presence of a lower number of olfactory lamellae (less than 17), a unique condition in *Tetragonopterus* (Silva et al., in preparation). Our results revealed that *T. araguaiensis* is not related to *T. anostomus*, as one could expect, in which both share the presence of 18–20 gill rakers on the lower limb of the branchial arches (Silva et al., 2013). Such feature is then interpreted as homoplastic and may be linked to the same feeding strategies adopted by these sympatric species inhabiting the Rio Araguaia of the Amazon basin.

4.2. Timing of Tetragonopterinae evolution and historical biogeography

Our relaxed molecular clock tree based on the available fossil record (Fig. 3) reveals a Late Oligocene/Early Miocene origin for diversification within *Tetragonopterus* (~18 Ma). Hydrographic dynamic across the Amazon basin during the Miocene (24–6 Ma) is well documented (Hoorn et al., 2010; Wesselingh and Hoorn, 2011 and references therein). Most notably, the Pebas system was predominantly dominated by lakes and lowland areas (Hoorn, 1993), which served as home for most Amazonian fishes that were able to move freely over almost the entire western Amazon (Wesselingh and Hoorn, 2011). Additionally, the Purus arch divided westerly and easterly drainages until the Late Miocene (Figueiredo et al., 2009) with cratonic rivers of the Guianese and Brazilian shields draining eastward (Harris and Mix, 2002; Figueiredo et al., 2009). This could potentially suggest that these major isolations promoted the first two contemporaneously diversifications within *Tetragonopterus* splitting *Moenkhausia georgiae* and then *T. rarus* from the Guiana Shield to the clade with remaining species during the Early–Middle Miocene.

Our dated phylogeny shows that subsequent cladogenetic events occurred mostly during the Middle and Late Miocene at 13–6 Ma (Fig. 3). This time coincides with the establishment of an initial transcontinental drainage system with the formation of the main Amazon river channel (Figueiredo et al., 2009) through the uplift of mountain chains in the Central and Northern Andes (~12 Ma) that may have promoted events of allopatric speciation and extinction (Hoorn et al., 2010). The La Venta formation in the present Magdalena Valley of Colombia, for example, contains deposits from the Middle Miocene (13.5–11.5 Ma) with numerous fossils of Neotropical fishes with living relatives (Lundberg et al., 1998). The combination of strong geological activity during that time may have influenced cladogenetic events within *Tetragonopterus*. More recently, species diversification within *Tetragonopterus* occurred during the Pliocene (~5–2.5 Ma) when climate oscillation influenced sea level fluctuations and transgression across South America (Hubert and Renno, 2006; Hubert et al., 2007), despite this relatively recent marine incursion hypothesis remains in debate (Bloom and Lovejoy, 2011). Sedimentological and palynological data was used to propose two major marine incursions, first occurring 20–17 Ma and the second 12–10 Ma (Hoorn, 1993; Hoorn et al., 2010). Within this context, we suggest that Miocene transgressions played an important role on the colonization with subsequent species diversification in *Tetragonopterus* dwelling in the Brazilian Shield of the Tocantins-Araguaia, São Francisco, Tapajós and Xingu basins through ancient wetlands of the Paranense Sea (Lundberg et al., 1998; Lima and Ribeiro, 2011).

Another interesting result is the timing of diversification within the *Tetragonopterus argenteus*. It is widely distributed throughout most of the South American drainages, being the single *Tetragonopterus* species occurring in the Paraná–Paraguay system. We found that the specimen from the Río Paraguay split from that one from the Río Marañon in the Peruvian Amazon during the Quaternary at approximately 1.7 Ma (Fig. 3). Interestingly, phylogeographic studies with Neotropical fishes have revealed similar results. For example, Sivasundar et al. (2001) found that *Prochilodus nigricans* from the Amazon basin split from *P. lineatus* from the Paraguay basin at around 4–2 Ma. Similarly, Hubert et al. (2007) used a molecular clock analysis of mtDNA and estimated a separation between specimens of *Pygocentrus nattereri* from the upper Amazon and upper Paraná–Paraguay basins at around 1.8 Ma. These coincident diversification can be explained by the origin of the Pantanal Wetland by tectonic reactivations of Precambrian faults at around 2.5 Ma (Soares et al., 1998; Assine, 2004), as hypothesized for Neotropical rheophilic fishes (Ribeiro et al., 2013). Such event likely promoted the subsequent colonization of the lineage of *T. argenteus* living nowadays in the Paraguay and lower Paraná basins.

Overall, our molecular dated phylogeny depicts the interspecific relationships within *Tetragonopterus* that can permit future research on the evolutionary processes modulating species diversification within the genus. Our results also corroborate the molecular hypothesis of the composition of Tetragonopterinae exclusively by *Tetragonopterus* and its placement as close related to Characinae and relatives. Further ecological aspects should be addressed to understand the variety of morphological features present in distinct and diverse monophyletic lineages of *Tetragonopterus* presented herein.

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