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Phylogeny and biogeography of Triportheidae (Teleostei: Characiformes) based on molecular data ☆

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ABSTRACT

Triportheidae represents a relatively small family of characiform fishes with highly modified morphology. The relationship among characiform families is still unclear, and a phylogenetic analysis for the family including a representative number of *Triportheus* species has never been performed. Here, we inferred a phylogeny for 19 of the 22 species recognized for this family and two possible new *Triportheus* species using two mitochondrial and three nuclear genes. Our results show that (1) Triportheidae is monophyletic and a sister group of the clade consisting of the families Bryconidae and Gasteropelecidae; (2) *Triportheus* is monophyletic, but some species need to be reviewed and described; (3) all genera in Triportheidae, except for *Agoniates* originated in the period between Early Oligocene and Early Miocene; and (4) speciation in Triportheidae coincides with important geological events in South America, reinforcing the importance of time-calibrated trees to study fish evolution.

1. Introduction

The order Characiformes contains about 2091 species distributed among 19 exclusively Neotropical families and four exclusively African families (Arroyave and Stiassny, 2011; Oliveira et al., 2011; Eschmeyer and Fong, 2015). Many taxonomic studies of this order have been performed, with the description of 309 species (approximately 15% of the total number of species) in the last 10 years (Eschmeyer and Fong, 2015). Oliveira et al. (2011) recognized Triportheidae as a valid characiform family and included in it the genera *Agoniates* (two species), *Clupeacharax* (one species), *Engraulisoma* (one species), *Lignobrycon* (one recent species) and *Triportheus* (17 species).

Malabarba (1998) found eight synapomorphies supporting a close relationship between *Triportheus* and *Lignobrycon*, but until now no additional morphological studies involving the remaining genera were published. Although this is a small group of characiforms, it contains highly modified species and its phylogenetic position among Characiformes remains unclear. Previous molecular studies suggested that Triportheidae could be the sister group of Gasteropelecidae or Bryconidae (Fig. 1), but in all of these studies, only a few specimens of *Triportheus*, the largest Triportheidae genus, were analyzed.

Considering the lack of information about the phylogenetic position of the Triportheidae among the Characiformes, two mitochondrial and three nuclear genes were sequenced for 19 of the 22 species currently recognized for this family and for representatives of all other Characiformes families. Therefore, the main goal of the present study was to formulate a hypothesis of the relationships among the Triportheidae species and genera and between this family and the other characiform families. In addition, a time-calibrated tree was inferred to investigate the temporal relationships between the origin of the Triportheidae species and genera and the main geological events in South America such as the formation of Magdalena, Amazonas, Paraguay, and São Francisco River basins.

2. Materials and methods

2.1. Taxa sampling and delineation of the ingroup and outgroup

The ingroup was composed of 32 specimens including representatives of 19 of 22 recognized Triportheidae species and two putative new, here identified as *Triportheus* aff. *rotundatus* and *Triportheus* sp. (Table 1). To test the position of Triportheidae in the Characiformes evolutionary tree, we used as outgroup the species employed by Oliveira et al. (2011) in their broad study of

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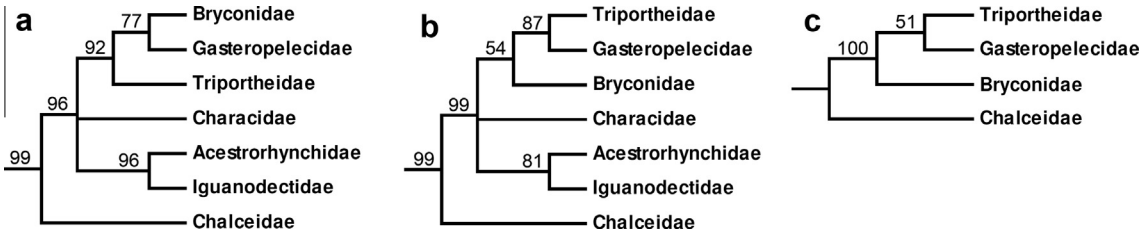

Fig. 1. Maximum likelihood trees showing the relationships among Triportheidae, Bryconidae and Gasteropelecidae according to Oliveira et al. (2011) (a), Abe et al. (2014) (b), and Abe et al. (2013) (c). Numbers represent bootstrap values obtained in 1000 pseudoreplicates.

Characidae relationships with additional data from Gasteropelecidae (Abe et al., 2013) and Bryconidae (Abe et al., 2014). All specimens for this study were collected in accordance with Brazilian laws under a permanent scientific collection license in the name of Dr. Claudio Oliveira (IBAMA-SISBIO, 13843-1). Additionally, this survey was carried out in strict accordance with the recommendations for the National Council for the Control of Animal Experimentation and the Federal Board of Veterinary Medicine. The studied material was deposited in the Laboratório de Biologia e Genética de Peixes (LBP), Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo, Brazil.

2.2. Molecular data collection

Total DNA was extracted from ethanol-preserved muscle samples using the DNeasy Tissue Extraction Kit (Qiagen) following the manufacturer's instructions. Partial sequences of the mitochondrial genes 16SrRNA and Cytochrome *b* (CytB) and the nuclear genes recombination activating gene 1 (Rag1), recombination activating gene 2 (Rag2) and myosin heavy chain 6 cardiac muscle alpha (Myh6) were amplified by polymerase chain reaction (PCR) with the primers listed in the Supplementary Material 1. Amplifications were performed in a total volume of 25 μl consisting of 2.5 μl 10X buffer (10 mM Tris–HCl, 15 mM $MgCl_2$), 0.5 μl $MgCl_2$ (50 mM), 0.5 μl each primer (5 μM); 0.4 μl of dNTPs (200 nM of each), 0.2 μl Taq Platinum polymerase (Invitrogen; 5 U/μl), 1 μl template DNA (10–50 ng) and 19.4 μl ddH_2O. The thermocycler profile used for the fragments 16SrRNA and CytB consisted of 35 cycles of 30 s at 95 °C, 45–120 s at 50–55 °C, and 90 s at 72 °C. Nested PCR was used to amplify the nuclear genes Rag1, Rag2 and Myh6. Amplification conditions for these genes in both rounds of PCR consisted of 15 cycles of 30 s at 95 °C, 45 s at 56 °C (according to primer), and 30 s at 72 °C followed by 15 cycles of 30 s at 95 °C, 45 s at 54 °C (according to primer), and 90 s at 72 °C. PCR products were purified using ExoSap-IT® (USB Corporation), sequenced using the Big DyeTM Terminator v 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems), purified again by ethanol precipitation and loaded into an automatic sequencer 3130 Genetic Analyzer (Applied Biosystems) at Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo, Brazil. Contigs were assembled and edited in BioEdit 7.0.9.0 (Hall, 1999). In cases of unclear nucleotide identity, IUPAC ambiguity codes were applied. All obtained sequences were deposited in GenBank (Table 1).

2.3. Alignment and phylogenetic analyses

Sequences of each gene were aligned using the MUSCLE algorithm under default parameters (Edgar, 2004), and the alignments were inspected by eye for any obvious misalignments that were subsequently corrected. A quality control step was included in our workflow as described in Oliveira et al. (2011). Genetic distances among sequences were calculated in Mega 5.04 (Tamura et al., 2011). To evaluate the occurrence of substitution saturation, we estimated the index of substitution saturation (Iss) in DAMBE 5.2.31 (Xia and Xie, 2001) as described in Xia et al. (2003) and Xia and Lemey (2009).

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 AICc (Akaike Information Criterion, corrected for finite sample sizes). The best-fit model of nucleotide substitution was searched in Mega 5.04 (Tamura et al., 2011) under default parameters using the AICc (see Posada and Buckley, 2004, for justification).

RAxML (Stamatakis, 2006) running in the web servers RAxML-HPC2 on TG (Stamatakis et al., 2008; Miller et al., 2010) was used for all maximum likelihood analyses with a mixed partition model. Random starting trees were run for each independent ML tree search, and all other parameters were set to default values. All ML analyses were conducted following the 13 partition scheme as suggested by the AICc. Topological robustness was investigated using 1000 non-parametric bootstrap replicates.

The estimation of divergence times in the ML phylogeny was carried out using BEAST (Bayesian evolutionary analysis sampling trees) 1.8.0 (Drummond et al., 2012) on a reduced dataset that included the family Triportheidae and representatives of the families Gasteropelecidae, Bryconidae and Characidae. To calibrate our molecular clock, we followed the guideline proposed by Parham et al. (2012). The fossil species *Lignobrycon ligniticus* (Woodward, 1898) (type specimen: BMNH P9012) was studied by Malabarba (1998), who showed that *L. ligniticus* is the sister group of *L. myersi* (a species included in our phylogeny) and that these two species are the sister group of *Triportheus*.*Lignobrycon ligniticus* was described based on complete specimens collected in the Tremembé Formation, Taubaté Basin, São Paulo, Brazil. Geological studies (Riccomini et al., 1991) confirmed the age of this formation as Oligocene, as also suggested by studies in mammalian fossils (Soria and Alvarenga, 1989) and pollens (Lima et al., 1985). According to the International Commission on Stratigraphy (www.stratigraphy.org), the Oligocene extended from 33.90 to 23.03 million years ago (Myr). These dates were implemented in BEAST v.1.8.0 (Drummond et al., 2012) with a log-normal prior offset of 28.5 and standard deviation of 1.8. We used a lognormal relaxed clock (uncorrelated) model and a birth–death process to calibrates our phylogenetic tree. Furthermore, we used the GTR (Generalized time-reversible, Tavaré, 1986) for nucleotide substitution model. All clade-age estimates are presented as the mean and 95% highest posterior density (HPD) values. The analysis was run for 20 million generations and sampled every 1000th generation. The tree obtained from RAxML analysis was used as an initial tree for the Bayesian inference. Stationarity and sufficient mixing of parameters (ESS > 200) were checked using Tracer 1.5 (Rambaut and Drummond, 2007a). All sampled topologies beneath the asymptote (5,000,000 generations) were discarded as part of a burn-in procedure. The remaining trees were used to built a 50% majority-rule consensus tree using TreeAnnotator v.1.8.0 (Rambaut and Drummond, 2007b).

Table 1
Triportheidae species analyzed in the present study. Asterisks indicate specimens sequenced in Oliveira et al. (2011).

Species	Voucher	Specimen	Locality	Geographic position	16S	CytB	Myh6	Rag1	Rag2
*Agoniates anchovia**	LBP6740	33,471	Lago Catalão/Manaus/AM/Brazil	S03°09.761′ W59°54.487′	HQ171378	HQ289665	–	–	HQ289472
*Agoniates halecinus**	LBP5503	26,594	Igarapé Uiratapuru/Laranjal do Jari/AP/Brazil	S00°34′03″ W52°34′41″	HQ171342	HQ289631	HQ289051	HQ289280	HQ289437
*Engraulisoma taeniatum**	LBP4038	22,896	Rio Moa/Cruzeiro do Sul/AC/Brazil	S7°37′20.0″ W72°47′42.2″	HQ171337	HQ289626	HQ289046	HQ289239	HQ289433
*Engraulisoma taeniatum**	LBP4038	22,897	Rio Moa/Cruzeiro do Sul/AC/Brazil	S7°37′20.0″ W72°47′42.2″	HQ171299	HQ289588	HQ289009	–	–
*Clupeacharax anchoveoides**	LBP5046	26,012	Lagoa Bairro Caiçara/Cáceres/MT/Brazil	S16°06′56′ W 57°44′33″	HQ171300	HQ289589	HQ289010	–	–
*Lignobrycon myersi**	LBP8094	37,519	Rio do Braço/Ilhéus/BA/Brazil	S14°41′11.7′ W39°16′28.0″	HQ171402	HQ289689	HQ289110	HQ289303	–
Triportheus albus	LBP8806	44,141	Lagoa do Japonês/Rio Araguaia/Cocalinho/MT/Brazil	S13°26′19.4′ W50°39′46.4″	KT281182	KT281198	KT281226	KT281244	KT281267
Triportheus albus	LBP8806	44,144	Lagoa do Japonês/Rio Araguaia/Cocalinho/MT/Brazil	S13°26′19.4′ W50°39′46.4″	KT281183	KT281212	KT281225	KT281258	KT281282
Triportheus angulatus	LBP4213	22,743	Rio Juruá/Cruzeiro do Sul/AC/Brazil	S07°09′49.6′ W73°43′29.7″	KT281174	KT281196	KT281232	KT281242	KT281265
Triportheus angulatus	LBP10593	49,353	Igarapé São Francisco/Rio Branco/AC/Brazil	S09°56′16.6″ W67°52′48.6″	KT281188	KT281207	KT281233	KT281253	KT281277
Triportheus auritus	LBP3052	19,160	Rio Orinoco/Caicara del Orinoco/Bolivar/Venezuela	N07°38′11.6″ W66°19′04.2″	KT281169	KT281193	KT281217	KT281239	KT281263
Triportheus brachipomus	LBP4426	24,305	Rio Negro/Barcelos/AM/Brazil	S00°55′30.0″ W62°51′51.4″	KT281175	KT281204	KT281227	KT281250	KT281273
Triportheus brachipomus	LBP10167	47,590	Rio Apure/Cabruta/Guárico/Venezuela	N07°37′24.4′ W66°24′48.0″	KT281187	KT281206	KT281221	KT281252	KT281275
Triportheus culter	LBP11920	22,709	Rio Juruá/Cruzeiro do Sul/AC/Brazil	S07°09′49.6′ W73°43′29.7″	KT281173	KT281214	KT281220	KT281260	KT281276
Triportheus guentheri	LBP10293	42,910	Baia da Inhuma/Rio São Francisco/São Roque de Minas/MG/Brazil	S20°11′03.4′ W45°50′57.9″	KT281180	KT281195	KT281224	KT281241	KT281264
Triportheus guentheri	LBP10293	47,240	Baia da Inhuma/Rio São Francisco/São Roque de Minas/MG/Brazil	S20°11′03.4′ W45°50′57.9″	KT281186	KT281203	KT281226	KT281249	KT281272
Triportheus magdalenae	LBP18993	75,307	Rio Coelho/Rio Magdalena/Coelho/Tolima/Colombia	N04°17′25.4″ W74°53′28.1″	KT281192	KT281216	–	KT281262	–
Triportheus magdalenae	LBP18993	75,308	Rio Coelho/Rio Magdalena/Coelho/Tolima/Colombia	N04°17′25.4″ W74°53′28.1″	KT281190	KT281215	KT281237	KT281261	KT281284
*Triportheus nematurus**	LBP39	3503	Rio Miranda/Corumbá/MS/Brazil	S19°34.630′ W57°01.123′	HQ171383	HQ289670	HQ289091	HQ289284	HQ289476
*Triportheus orinocensis**	LBP2663	15,580	Laguna de Castilleros/Caicara del Orinoco/Bolivar/Venezuela	N07°30′50.9″ W66°09′19.8″	HQ171253	HQ289544	HQ288963	HQ289160	HQ289351
Triportheus orinocensis	LBP9980	43,125	Laguna de Castilleros/Caicara del Orinoco/Bolivar/Venezuela	N07°30′50.9″ W66°09′19.8″	KT281181	KT281211	KT281223	KT281257	KT281281
Triportheus pantanensis	LBP8445	41,694	Lagoa Marginal/Rio Cuiabá/Cáceres/MT/Brazil	S16°03′13.6′ W57°48′31.8″	KT281179	KT281200	KT281228	KT281246	KT281269
Triportheus pantanensis	LBP9251	44,492	Rio Paraguay/Cáceres/MT/Brazil	S16°05′14.8″ W57°42′17.8″	KT281184	KT281202	KT281229	KT281248	KT281271
Triportheus rotundatus	LBP2840	17,042	Lago do Silência/Boca do Acre/AM/Brazil	S08°51′21.5″ W68°42′22.6″	KT281171	KT281199	KT281219	KT281245	KT281268
Triportheus aff. rotundatus	LBP11917	45,312	Rio Miranda/Miranda/MS/Brazil	S19°34′58.3′ W57°01′18.9″	KT281185	KT281201	KT281231	KT281247	KT281270
Triportheus signatus	LBP5534	27,202	Rio Balsas/Balsas/MA/Brazil	S 07°32′26′ W46°02′21′	KT281176	KT281209	KT281222	KT281255	KT281279
Triportheus signatus	LBP6554	31,049	Rio Piracicaba/Santa Maria da Serra/SP/Brazil	S22°38′23.1″ W48°03′11.0″	KT281178	KT281210	KT281230	KT281256	KT281280
Triportheus trifurcatus	LBP5750	28,111	Rio Araguaia/Aragarças/GO/Brazil	S15°53′31.5″ W52°15′02.0″	KT281177	KT281208	KT281234	KT281254	KT281278
Triportheus trifurcatus	LBP8775	44,058	Lagoa do Chico/Rio Araguaia/Cocalinho/MT/Brazil	S13°19′22.8′ W 50°37′20.7″	KT281191	KT281194	KT281218	KT281240	–
Triportheus venezuelensis	LBP2194	15,523	Laguna de Castilleros/Caicara del Orinoco/Bolivar/Venezuela	N07°30′50.9″ W66°09′19.8″	KT281170	KT281205	KT281236	KT281251	KT281274
Triportheus venezuelensis	LBP11918	19,155	Rio Orinoco/Caicara del Orinoco/Bolivar/Venezuela	N07°38′11.6″ W66°19′04.2″	KT281172	KT281213	–	KT281259	KT281283
Triportheus sp. nov.	LBP15930	63,501	Lagoa Margina/Rio Xingu/Canarana/MT/Brazil	S13°31′02.8″ W53°04′41.8″	KT281189	KT281197	KT281235	KT281243	KT281266

2.4. Estimation of ancestral ranges

Data on the geographic distributions of species in the Triportheidae were taken from the original species descriptions and information available from the online Catalog of Fishes (Eschmeyer and Fong, 2015). To maintain consistency with previous biogeographic analyses of the family Characiformes we assigned taxa to geographic areas with modifications of ecoregion classifications of Vari (1988) and Vari and Malabarba (1998), which were defined by patterns of endemism in the Neotropical fishes. Our samples were drawn from the following eight ecoregions: (Region A) East Coastal Drainages, (Region B) Tocantins and Araguaia Basins, (Region C) São Francisco Basin, (Region D) Paraguay and Lower Paraná Basins, (Region E) Amazon Basin, (Region F) Orinoco and North Coastal Drainages, (Region G) Magdalena Basin, and (Region H) Parnaíba Basin.

We estimated likelihoods of ancestral range evolution using the Dispersal-Extinction-Cladogenesis (DEC, Ree and Smith, 2008; DEC + J, Matzke, 2013a), Bayarealike and Bayarealikej (both modified from Landis et al., 2013) models of geographic (species) range evolution. The four models of range evolution (i.e. DEC, DEC + J, Bayarealike, and Bayarealikej) were implemented in the R package BioGeoBEARS (Matzke, 2013b). Additionally, five scenarios (called M0 to M4) with values of dispersal rate between adjacent areas of 0.50 and 1.00 and dispersal rate between no adjacent areas with values of 1.00, 1.00E−01, 1.00E−04, and 1.00E−20 were tested. Global likelihoods of biogeographic scenarios using the four models were compared using the Akaike Information Criterion (AIC) (Akaike, 1973).

3. Results

Partial sequences of two mitochondrial (16SrRNA and CytB) and three nuclear genes (Myh6, Rag1 and Rag2) were obtained for 294 specimens, 26 of which were sequenced in the present study (Table 1). Two matrices were constructed. Matrix 1 included data from all specimens (identified as the Characiformes matrix) containing 4699 bp, and Matrix 2 included representatives from the Bryconidae, Gasteropelecidae, Triportheidae and selected Characidae (identified as Triportheidae) data totaling 4666 bp. These matrixes were deposited in TreeBase (www.treebase.org) under the number 17956.

Missing data due to problems with PCR experiments, sequencing, or entries that were not available in GenBank corresponded to 9.7% of Matrix 1 and to 9.1% of Matrix 2 (Table 2). Missing data were more prevalent among nuclear loci (16.1% and 9.1% for Matrix 1 and 2, respectively) than mitochondrial genes (9.4% and 9.0% for Matrix 1 and 2, respectively), possibly due to non-conserved priming regions and a higher risk of cross-contamination in the nested PCR procedure (a few sequences were eliminated from the matrix after contamination was diagnosed by our quality-control protocol). For each matrix and gene, the number and percentage of sequences obtained, size (bp), number of variable sites, base pair composition, overall mean genetic distance (*p*-distance), best substitution model for the gene, (shape) parameter of the C distribution, proportion of invariant (I) sites, number of informative characters under parsimony, and proportion of informative characters under parsimony are presented in Table 2. The overall mean genetic distance observed was between 0.083 ± 0.005 (Myh6) and 0.215 ± 0.007 (CytB) (Matrix 1) and between 0.062 ± 0.005 (Myh6) and 0.209 ± 0.007 (CytB) (Matrix 2), suggesting that the analyzed sequences exhibited sufficient genetic variation for phylogenetic analyses of species, genera and families. All data were further tested to investigate the occurrence of substitution saturation (Xia et al., 2003; Xia and Lemey, 2009), and the results showed no significant saturation. The best-fitting model of nucleotide substitution calculated for each gene is shown in Table 2. The combined data set contains significant phylogenetic information, as most major lineages along the backbone of the tree were supported by high bootstrap values.

Table 2
Information content and characteristics of each molecular data partition. The upper line represents the values of Matrix 1 (total dataset), and the lower line represents the values of Matrix 2 (Triportheidae + Bryconidae + Gasteropelecidae + select Characidae).

	Gene					
	16S	CytB	Myh6	Rag1	Rag2	Total
Number of sequences	294 (100%)	269 (91.5%)	245 (83.3%)	238 (80.9%)	257 (87.4%)	294
	101 (100%)	96 (95.0%)	86 (85.1%)	81 (80.2%)	95 (93.1%)	101
bp after alignment	653	992	755	1265	1034	4699
	626	992	752	1265	1031	4666
Number of variable sites	409	693	397	896	706	3101
	314	593	282	617	461	2267
Number of informative characters under parsimony	333	589	330	701	592	2545
	242	513	221	407	325	1708
% Informative characters under parsimony	50.9	59.3	43.7	55.4	56.2	54.2
	38.7	51.7	29.4	32.2	31.5	36.6
Π_T	0.226	0.297	0.247	0.226	0.232	0.246
	0.225	0.295	0.246	0.226	0.232	0.246
Π_C	0.236	0.291	0.213	0.240	0.251	0.249
	0.235	0.286	0.213	0.242	0.252	0.249
Π_A	0.314	0.266	0.307	0.253	0.245	0.271
	0.315	0.270	0.306	0.257	0.244	0.273
Π_G	0.224	0.146	0.234	0.281	0.272	0.234
	0.225	0.149	0.234	0.276	0.271	0.233
Overall mean genetic distance (*p*-distance)	0.124 ± 0.007	0.215 ± 0.007	0.083 ± 0.005	0.108 ± 0.004	0.107 ± 0.004	0.135 ± 0.003
	0.108 ± 0.007	0.209 ± 0.007	0.062 ± 0.005	0.075 ± 0.004	0.069 ± 0.004	0.112 ± 0.003
Nucleotide substitution model	GTR + I + Γ	GTR + I + Γ	T92 + I + Γ	K2P + I + Γ	K2P + I + Γ	GTR + I + Γ
	GTR + I + Γ	GTR + I + Γ	T92 + Γ	T92 + Γ	K2P + Γ	GTR + I + Γ
α (shape) parameter of Γ distribution	0.58	0.65	1.03	0.88	0.92	0.63
	0.63	1.09	0.33	0.33	0.48	0.56
Proportion of invariants (I) sites	0.27	0.29	0.42	0.21	0.23	0.28
	0.29	0.36	–	–	–	0.29

Table 3
Comparison of log likelihoods and AIC and BIC values among different partitioning schemes (from 1 to 13 partitions) for Matrix 1 (all data) and Matrix 2 (Triportheidae + Bryconidae + Gasteropelecidae + selected Characidae). For each type of analysis, the following results are shown: total number of parameters, log likelihood calculated using RAxML (L_{ML}), AIC values, the difference in AIC values among model i and the best model (Δi = AICi – AICmin), and BIC_{ML} values.

Number of partitions[a]	Number of parameters	L_{ML}	AIC	Delta$_i$	BIC_{ML}
Matrix 1					
1	9	196068.217	392154.434	10095.659	392169.482
2	19	195317.812	390673.625	8614.850	390705.393
4A	39	195525.437	391128.874	9070.099	391194.082
4B	39	195264.504	390607.008	8548.233	390672.216
5	49	195152.445	390402.891	8344.116	390484.819
13	129	190900.388	382058.775	0.000	382274.464
Matrix 2					
1	9	56036.502	112091.005	1750.540	112106.053
2	19	55884.183	111806.366	1465.901	111838.134
4A	39	55932.866	111943.731	1603.267	112008.939
4B	39	55854.879	111787.759	1447.294	111852.967
5	49	55753.934	111605.869	1265.405	111687.797
13	129	55041.232	110340.464	0.000	110556.153

[a] 1 Partition = whole dataset; 2 partitions = mitochondrial (16S + CytB) and nuclear (Myh6 + Rag1 + Rag2); 4 partitions A = 16S and 1st, 2nd, and 3rd codon position of protein coding genes; 4 partitions B = 16S + CytB and 1st, 2nd, and 3rd codon position of nuclear genes; 5 partitions = by each gene (16S + CytB + Myh6 + Rag1 + Rag2); 13 partitions = 16S + each codon position of each protein coding gene (1st, 2nd, and 3rd codon position of CytB; 1st, 2nd, and 3rd codon position of Myh6; 1st, 2nd, and 3rd codon position of Rag1; 1st, 2nd, and 3rd codon position of Rag2).

Six different partitioning schemes ranging from 1 to 13 partitions were tested to establish the optimal number of data partitions (following Li et al., 2008) for the final analysis for each matrix. The results showed that the 13 partition model was the best choice (Table 3). However, ML analysis conducted with the other partitioning schemes produced the same final topology, with minor differences in branch length and support values (data not shown).

3.1. Phylogenetic relationships of the Triportheidae

As shown in Fig. 2, Triportheidae is monophyletic and a sister group of Gasteropelecidae and Bryconidae. Within Triportheidae, *Lygnobrycon myersi* is the sister group of the remaining members of the family (Fig. 3). A second well-supported group is composed of the two *Agoniates* species, sister-groups of *Clupeacharax anchoveoides,* and both as sister-group of *Engraulisoma taeniatum* (Fig. 3). The third group is composed of all sampled species of *Triportheus*, which supports the monophyletic status of this genus (Fig. 3).

3.2. Estimates of divergence times of Triportheidae clades

We found that the mean substitution rate for the Triportheidae dataset as estimated using BEAST was 0.001847 substitution/site/Myr. The origin of Triportheidae was estimated at 38.9 ± 10.2 Myr (Fig. 3). Within the Triportheidae, *Lignobrycon* lineage originated at 29.2 ± 3.4 Myr, *Engraulisoma* lineage originated at 22.1 ± 6.8 Myr, *Clupeacharax* and *Agoniates* lineages originated at 15.9 ± 7.1 Myr, and *Triportheus* lineage originated at 26.2 ± 6.5 Myr (Fig. 3).

3.3. Historical biogeography

The results for the five biogeographic scenarios tested with BayArea and DEC models show that the DEC + J model with dispersal rate between adjacent areas = 1.00 and dispersal rate between no adjacent areas = 1.00E−01 results in the better hypothesis of ancestral range estimation (Table 4). The better hypothesis suggests that the Triportheidae ancestor was widespread through the Regions A, D, and E at 29.2 ± 3.4 Myr (Fig. 4) and at 26.2 ± 6.5 Myr this ancestor was extinct from Region D. Region B was colonized from Region E by an ancestor of *Triportheus auritus* at 20.7 ± 6.5 Myr, from Region F by an ancestor of *T. albus* at 8.9 ± 4.0 Myr, and by an ancestor of *T. trifurcatus* at 4.3 ± 1.9 Myr. Region C was colonized from Region F by an ancestor of *T. guentheri* at 12.4 ± 4.4 Myr. Region D was colonized again four times, tree from Region E, at 22.1 ± 6.8 Myr (*Engraulisoma* lineage), 15.9 ± 7.1 Myr (*Clupeacharax* lineage), and 4.3 ± 1.9 Myr (the ancestor of *T. pantanensis* and *T. angulatus*), and one from Region F at 9.5 ± 3.6 Myr. Region E was colonized from Region F by an ancestor of *T. albus* at 8.9 ± 4.0 Myr and by an ancestor of the lineage of *T. venezuelensis*, *T. pantanensis*, *T. angulatus*, *T. trifurcatus*, *Triportheus* sp. nov., and *T. signatus* at 6.8 ± 2.7 Myr. Region F was colonized from Region E by an ancestor of *T. auritus* at 20.7 ± 6.5 Myr, and by an ancestor of all *Triportheus* species investigated less *T. magdalenae*, *T. culter*, and *T. rotundatus* at 16.0 ± 5.7 Myr. Region G was colonized from Region E by an ancestor of *T. magdalenae* at 6.7 ± 4.6 Myr. Region H was colonized from Region E by an ancestor of *T. signatus* at 2.1 ± 1.4 Myr.

4. Discussion

This study represents the first phylogenetic analysis in which a significant number of *Triportheus* species along with all other Triportheidae genera were investigated. Our results indicate that the family Triportheidae is monophyletic, as proposed by Oliveira et al. (2011), as are the genera *Triportheus* and *Agoniates*.

Our results suggest that Triportheidae is a sister group of Bryconidae and Gasteropelecidae (Fig. 2). According to Weitzman (1954), the presence of expanded coracoids in *Triportheus* and the so-called subfamily Gasteropelecinae is likely due to convergent evolution, i.e., they arose independently in these groups. This view was adopted by other authors, including Castro and Vari (1990) and Mirande (2010). However, based on morphological analyses, Gregory and Conrad (1938) suggested that "*Chalcinus* (=*Triportheus*) is much the nearer to the structural ancestor of *Gasteropelecus*", a hypotheses rejected in the present study. The close relationship between Bryconidae and Gasteropelecidae as observed here has been suggested in previous studies using molecular markers (Oliveira et al., 2011; Abe et al., 2013) but was refuted in Abe et al. (2014), where Triportheidae appears as sister-group of Gasteropelecidae. Considering that the number of species of Triportheidae used in the study of Abe et al. (2014) was small

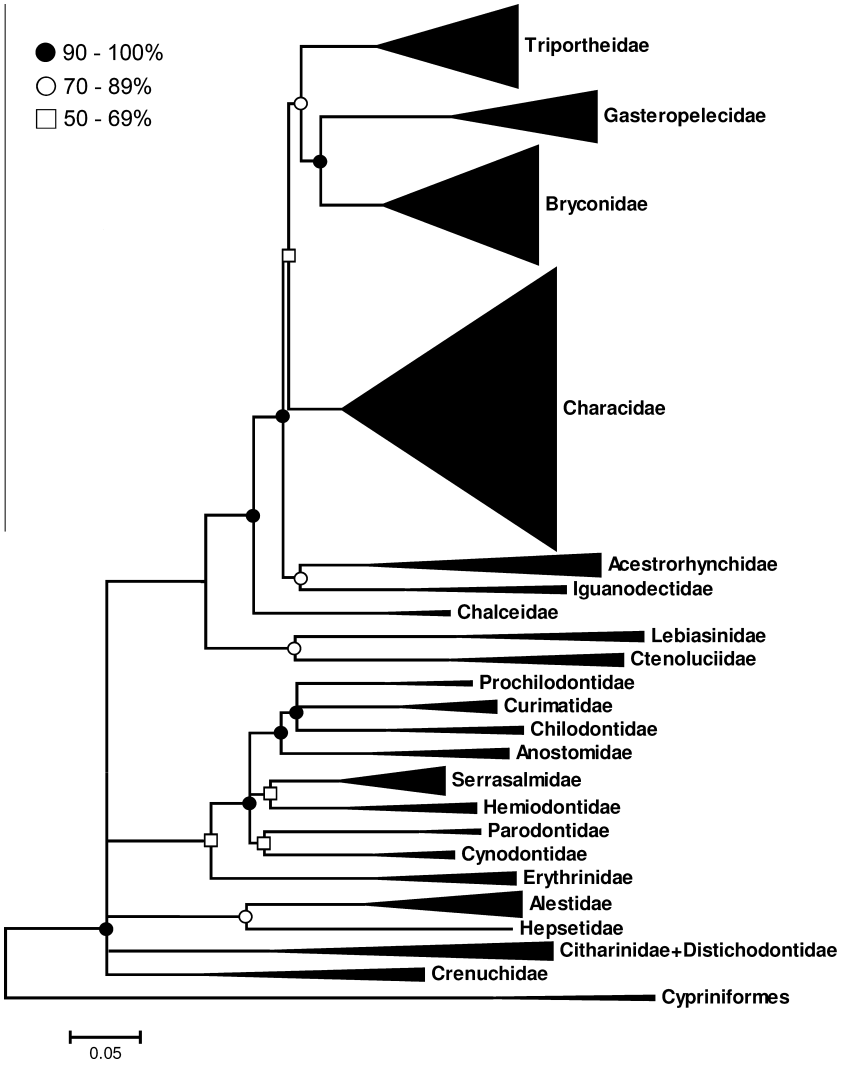

Fig. 2. Maximum likelihood tree obtained in the partitioned analysis showing relationships among Characiformes families.

compared with those of Bryconidae and Gasteropelecidae, this result may be due to missing data and taxon sampling artifacts. Further studies involving different markers will be necessary for a better understanding of the relationships of these three families.

In all analyses, *Lignobrycon myersi* (a single recent species) is the sister group of all remaining Triportheidae, as proposed by Oliveira et al. (2011). The remaining relationships inside Triportheidae proposed by Oliveira et al. (2011) were also corroborated in the present study, even with a significant increase of *Triportheus* samples. Malabarba (1998) found eight synapomorphies supporting a close relationship between *Triportheus* and *Lignobrycon*, but the relationships of these genera with the remaining Triportheidae were not investigated. Thus, it would be informative to revisit the morphological hypothesis within the context of the results arrived at herein.

4.1. Origin and diversification of the Triportheidae

The main lineages of Neotropical freshwater fishes were present in South America by the Late Cretaceous, and much of their diversification occurred before or during Paleogene (Lundberg et al., 1998; Albert et al., 2011; Lopez-Fernández and Albert, 2011). We estimated the origin of all genera in Triportheidae, except *Agoniates*, in the period corresponding to Early Oligocene to Early Miocene. According to Lopez-Fernández and Albert (2011), during the Oligocene, as had occurred before, major marine regressions exposed large areas of interior floodplains, allowing dramatic and sometimes rapid expansions of freshwater habitats including the formation of the so-called "foreland basin" (Lima and Ribeiro, 2011). These events could permit the rapid expansion of a primitive Triportheidae through the proto-Paraná and proto-Amazon systems as suggested in our biogeographical analysis (Fig. 4).

Within Triportheidae, the ancestor of all known genera lived in the Regions A (East Coastal Drainages), D (Paraguay and Lower Paraná Basins), and E (Amazon Basin) (Fig. 4), but only the lineage of *Lignobrycon* remained in the Region A until the present. The ancestor of all other genera lived originally in regions D and E but were soon extinct from Region D, which was recolonized more recently (Fig. 4).

The clade composed of *Engraulisoma, Clupeacharax* and *Agoniates* comprises species widely distributed in the Amazon and La Plata basins and the ancestral of this lineage originated at 26.2 ± 6.5 (Fig. 3) in the Amazon Basin and from this region its colonized the Paraguay and Lower Paraná Basins two times (Fig. 4). The first dispersion occurred at 22.1 ± 6.8 Myr (*Engraulisoma* lineage) and the second at 15.9 ± 7.1 Myr (*Clupeacharax* lineage).

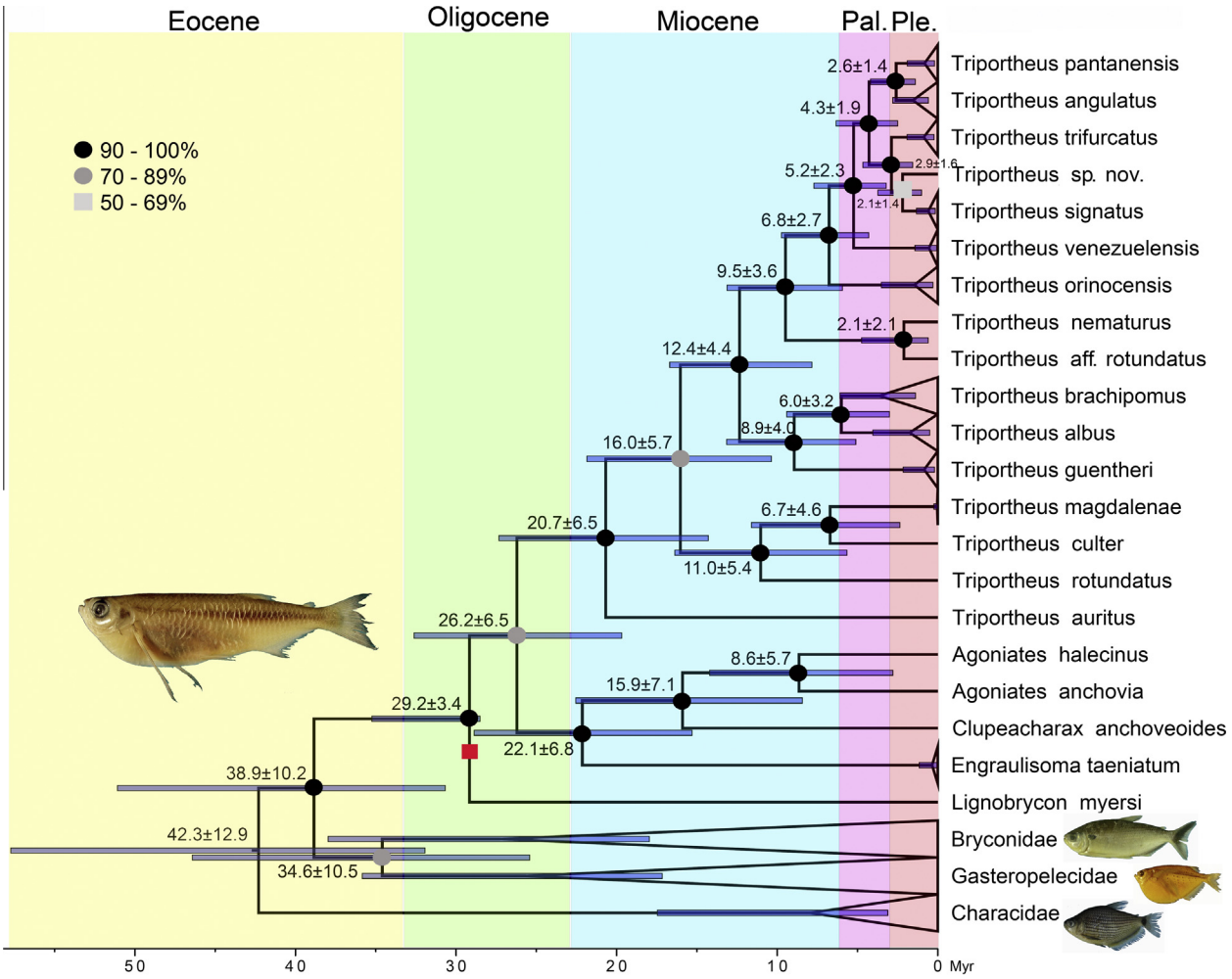

Fig. 3. Time-calibrated phylogeny obtained using BEAST from 15 million generations indicating the divergence over time of the family Triportheidae. The red square shows the calibration point based on the fossil of *Lignobrycon ligniticus* (28.5 ± 5.5 Myr). Scale = millions of years before present. Node bars = Node height highest posterior density (HPD) intervals at 95%. Node numbers = node ages ± Node height 95% HPD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The first event coincides with the rise of the Chapare Buttress that divided the paleo-Amazonas-Orinoco and Paraguay basins at 30–20 Myr (Lundberg et al., 1998) and the second event can be due to the shift south to the Michicola Arch at 11.8–10.0 Myr, when important headwater capture of Upper Paraguay by Amazonas occurred (Lundberg et al., 1998).

The ancestor of the genus *Triportheus* originated at 26.2 ± 6.5 Myr (Fig. 3). The first lineage to differentiate was that which gave origin to *T. auritus* at 20.7 ± 6.5 Myr. This species has the widest distribution in the genus (Malabarba, 2004). The ancestor originated in the Amazon Basin and dispersed to Tocantins and Araguaia Basins and Orinoco and North Coastal Drainages (Fig. 4).

Triportheus magdalenae (the single west Andean Triportheidae) from the Magdalena River is the sister group of *T. culter* from the Juruá River in Amazon (Fig. 3). These two species separated at 6.7 ± 4.6 Myr (Fig. 3) and *T. magdalenae* ancestor originated from an ancestor from Amazon Basin (Fig. 4). Abe et al. (2014), found that *Salminus* sp. from the Amazon basin and *S. affinis* from Magdalena River Basin separated at 4.1 ± 3.7 Myr, a similar value to that found here between *T. magdalenae* and *T. culter*. The most intense peaks of northern Andean mountain building occurred from the late middle Miocene (~12 Ma) and early Pliocene (~4.5 Ma) (Hoorn et al., 2010), which is in accordance with our results on the separation of these two species pairs. Abe et al. (2014) found that *Brycon moorei* from the Magdalena River basin separated from the other *Brycon* species at 21.3 ± 5.5 Myr. This old separation coincides with the first peak of mountain building in the Northern Andes (late Oligocene to early Miocene approximately at 23 Myr) (Hoorn et al., 2010). Thus, these results suggest that the Magdalene fish fauna diversified over a long period of time, a hypothesis that should be tested with additional data.

The ancestor of the species *Triportheus rotundatus* from the Amazon basin, the sister group of *T. magdalenae* and *T. culter*, originated at 11.0 ± 5.4 Myr (Fig. 3). This species is morphologically similar to the species we identified here as *T.* aff. *rotundatus* from the Paraguay River Basin, which is the sister group of *T. nematurus* that is also from the Paraguay River Basin (Fig. 3). These two groups belong to different lineages in *Triportheus* that separated at 16.0 ± 5.7 Myr (Fig. 3). This separation may be due to the rise of the Chapare Buttress at 30–20 Myr (Lundberg et al., 1998) or the shift south to the Michicola Arch at 11.8–10.0 Myr (Lundberg et al., 1998). This time is the limit of the age we found for the separation of the *T.* aff. *rotundatus* lineage from the *T. rotundatus* lineage, but the value is similar to that found by Abe et al. (2014) for the separation

Table 4
Maximum likelihood estimates for four biogeography scenarios indicates the DEC + J model range evolution best optimize (i.e. maximize likelihood) the geographical distribution of Triportheidae species under the model 4. Abbreviations: LEM, landscape evolution model; GRE, geographical range evolution; DEC, dispersal–extinction–cladogenesis; ln *L*, log likelihood; *K*, parameters; *d*, dispersal; μ, extinction; *j*, cladogenesis per-event weights; AIC, Akaike information criterion.

Scenarios	Model	Dispersal rate between adjacent areas	Dispersal rate between no adjacent areas	Ln *L*	*K*	*d*	μ	*j*	AIC	ΔAIC
M0	DEC	1.00	1.00E−20	−64.7829	2	0.0159	0.0049	0.0000	133.5659	25.4951
	DEC + J	1.00	1.00E−20	−58.1080	3	0.0086	0.0029	0.2444	122.2161	14.1453
	BAYAREALIKE	1.00	1.00E−20	−75.1165	2	0.0335	0.0909	0.0000	154.2330	46.1622
	BAYAREALIKE + J	1.00	1.00E−20	−59.0022	3	0.0064	0.0120	0.2227	124.0044	15.9336
M1	DEC	1.00	1.00	−67.3148	2	0.0071	0.0054	0.0000	138.6297	30.5589
	DEC + J	1.00	1.00	−52.7634	3	0.0025	0.0000	0.1115	111.5269	3.4561
	BAYAREALIKE	1.00	1.00	−76.3951	2	0.0108	0.0742	0.0000	156.7904	48.7196
	BAYAREALIKE + J	1.00	1.00	−55.4073	3	0.0026	0.0000	0.0888	116.8148	8.7440
M2	DEC	0.50	1.00E−04	−64.7785	2	0.0319	0.0049	0.0000	133.5571	25.4863
	DEC + J	0.50	1.00E−04	−57.3207	3	0.0136	0.0000	0.6568	120.6414	12.5706
	BAYAREALIKE	0.50	1.00E−04	−75.1097	2	0.0689	0.0921	0.0000	154.2194	46.1486
	BAYAREALIKE + J	0.50	1.00E−04	−58.5746	3	0.0128	0.0076	0.4659	123.1509	15.0801
M3	DEC	1.00	1.00E−04	−64.7807	2	0.0159	0.0049	0.0000	133.5615	25.4907
	DEC + J	1.00	1.00E−04	−57.6964	3	0.0075	0.0013		121.3929	13.3221
	BAYAREALIKE	1.00	1.00E−04	−75.1134	2	0.0338	0.0910	0.0000	123.5396	15.4688
	BAYAREALIKE + J	1.00	1.00E−04	−58.7697	3	0.0065	0.0099	0.2673	154.2270	46.1562
M4	DEC	1.00	1.00E−01	−64.1588	2	0.0140	0.0047	0.0000	132.3176	24.2468
	DEC + J	1.00	1.00E−01	−51.0354	3	0.0053	0.0000	0.2679	108.0708	0.0000
	BAYAREALIKE	1.00	1.00E−01	−74.2728	2	0.0259	0.0817	0.0000	112.5054	4.4346
	BAYAREALIKE + J	1.00	1.00E−01	−53.2527	3	0.0045	0.0015	0.2186	152.5456	44.4748

of *Brycon amazonicus* and *B. hilarii* (12.4 ± 5.8 Myr) and of *B. orbygnianus* and *B. gouldingi* (14.4 ± 6.3 Myr).

A second species pair from the Amazon and Paraguay basins is *Triportheus pantanensis* and *T. angulatus* (Fig. 3). This species pair separated at 2.6 ± 1.4 Myr, an event more recent than that discussed above. However, the formation of the Pantanal Wetland, where we found *T. pantanensis*, dated to approximately 2.5 Myr (Soares et al., 1998; Assine, 2004). The separation of the species and populations of Upper Paraguay and Amazon at this time were extensively discussed by Ribeiro et al. (2013) using *Jupiaba acanthogaster* as a model.

Four samples of *Triportheus* from the Orinoco River were analyzed. *Triportheus auritus*, as discussed above, is widely distributed in the Amazon Region and is the oldest living *Triportheus*. *Triportheus brachipomus* occurs in Orinoco and coastal rivers in north South America (Malabarba, 2004). Our analyses showed that a sample from the Negro River (Amazon Basin) could be morphologically and molecularly identified as *T. brachipomus*. The separation time between the samples from Orinoco and Negro River was 3.6 ± 2.4 Myr. These data are in accordance with the separation of the Amazon and the Orinoco with the raise of the Vaupes Arch (late Miocene) (Hoorn et al., 1995). These data are also similar to those found in the separation of a sample from *Brycon falcatus* from the Orinoco River and a second one from the Negro River (3.9 ± 3.3 Myr; Abe et al., 2014).

The remaining two endemic species of *Triportheus* from Orinoco, *T. orinocensis* and *T. venezuelensis* (Malabarba, 2004), are not sister groups (Fig. 3). The ancestor of *T. orinocensis* separated from the other *Triportheus* species at 6.8 ± 2.7 Myr, and the ancestor of *T. venezuelensis* separated from the other *Triportheus* species at 5.2 ± 2.3 Myr (Fig. 3). These times are earlier than those observed between *T. brachipomus* and *Brycon falcatus* samples (as discussed above) but are similar to those found by Mariguela et al. (2013) for specimens of *Hoplosternum littorale* and *Megalechis picta* from the Amazon and the Orinoco, between 11.7 to 6 Myr, and are also similar to the separation of *T. brachipomus* from *T. albus* (its sister group) that occurred at 6.0 ± 3.2 Myr (Fig. 3) suggesting that more than one event separated these two basins or that different species suffered speciation processes in different times.

Triportheus signatus is found in the Parnaíba River basin and some coastal rivers in northeastern Brazil (Malabarba, 2004). In the present study, we used a sample from a tributary of the Parnaíba River and a sample from the Tietê River (Upper Paraná Basin). The data showed that both are identical from the molecular and morphological points of view. This species has been identified as *T. nematurus* in the Upper Paraná River Basin (e.g. Langeani et al., 2007; Pavanelli et al., 2007) after the review of Malabarba (2004), but is recognized as introduced and even identified as *T. signatus* in some studies (e.g. Smith et al., 2007). Thus, based on our morphological and molecular analyses, we recognize *T. signatus* as occurring in the Upper Paraná River as an alien species.

Triportheus signatus is the sister group of a putative new species from the Xingu River, and both separated at 2.1 ± 1.4 Myr (Fig. 3). The biogeographical analyses showed that the ancestor of *T. signatus* originated from the Amazon Basin area (Fig. 4). The ichthyofauna of the Parnaíba River is composed of many endemic species (Ramos et al., 2014), but a relationship between species occurring in this river and in the Amazon Basin has been demonstrated (Vari, 1989). These two species are the sister group of *T. trifurcatus* (endemic of Tocantins-Araguaia Rivers – Malabarba, 2004) and separated from it at 2.9 ± 1.6 Myr (Fig. 3). The ancestor of *T. trifurcatus* also originated in the Amazon Basin region (Fig. 4).

The last group is composed of *Triportheus guentheri*, *T. brachipomus* (discussed above) and *T. albus* (Fig. 3). *T. guentheri* is the only species of this genus in São Francisco. *Triportheus albus* is found in Amazonas and the Araguaia-Tocantins Rivers (Malabarba, 2004). The ancestor of *T. guentheri* separated from the ancestor of *T. albus* and *T. brachipomus* at 8.9 ± 4.0 Myr (Fig. 3). Several authors have suggested that the fish fauna of the São Francisco basin is a hybrid combination of groups that occur in adjacent basins (Vari, 1988; Lima and Caires, 2011). Abe et al. (2014) found that *Brycon orthotaenia* (endemic of the São Francisco River) separated from the other related species at 17.1 ± 5.5 Myr, a time frame similar to that we found for *T. guentheri*.

In summary, our results show that: (1) Triportheidae is monophyletic and a sister group of Bryconidae and Gasteropelecidae; (2) *Triportheus* is monophyletic, but some species need to be reviewed and described; (3) all genera in Triportheidae, less *Agoniates*, originated in the period between Early Oligocene and Early

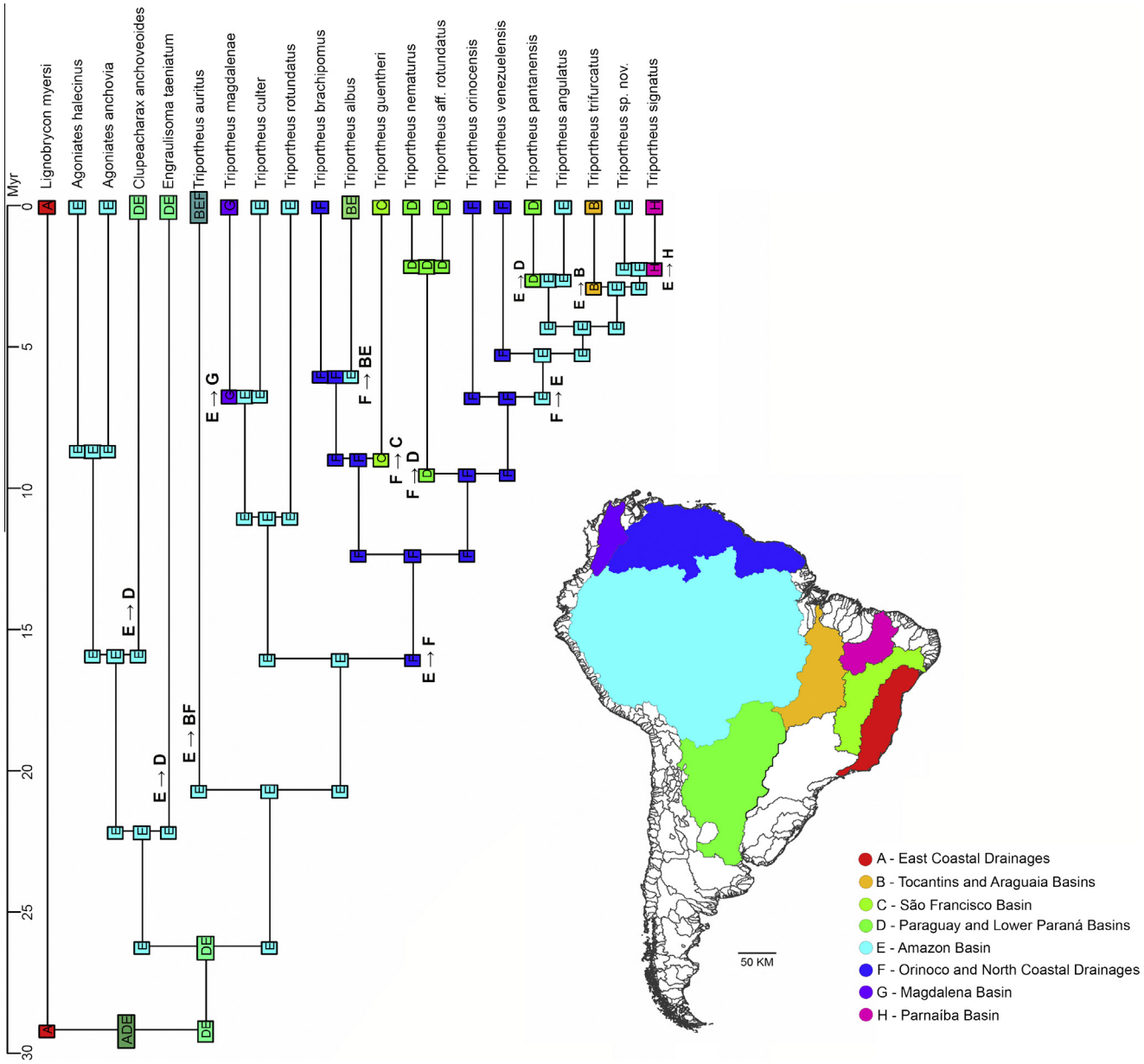

Fig. 4. Ancestral range estimation for Triportheidae using DEC + J model. Color changes between squares indicate dispersal and/or extinction events. Different colors represent species present in multiple geographical areas. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Miocene; and (4) speciation in Triportheidae coincides with important geological events in South America, reinforcing the use of time-calibrated trees to study fish evolution.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.11.018.

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