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Phylogeography and conservation genetics of the Amazonian freshwater stingray *Paratrygon aiereba* Müller & Henle, 1841 (Chondrichthyes: Potamotrygonidae)

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The family Potamotrygonidae is monophyletic comprising three genera: *Paratrygon* Duméril, *Potamotrygon* Garman and *Plesiotrygon* Rosa, Castello & Thorson. The distribution of most species in this family is restricted to a single basin or fluvial system. Only *Potamotrygon motoro*, *Potamotrygon orbignyi* and *Paratrygon aiereba* are found in more than one river basin. In this study we investigate genetic structuring of *Paratrygon aiereba*, from five rivers of the Amazon region: Negro, Solimões-Amazon-Estuary system, Tapajós, Xingu and Araguaia. Sixty-three individuals were sequenced for ATPase 6, and a representative subsample of 27 individuals was sequenced for COI. The COI dataset analysis indicated that *Paratrygon* is sister to all other potamotrygonid genera and species. Population parameters inferred from the analysis of ATPase 6 sequences revealed that the populations of this species are structured within each river, with no or nearly non-existent gene flow occurring between rivers and a positive correlation between geographic and genetic distances. *Paratrygon aiereba* is comprised of three geographically restricted clades with K2P interclade distances of at least 2%. Intraspecific divergence within *P. aiereba* is similar to the interspecific divergence observed in *Potamotrygon* spp. sampled throughout the same geographic area. Using the premises of COI barcoding and the allopatric distribution of the three *P. aiereba* clades, the taxon *P. aiereba* most likely comprises three distinct biological species. Since freshwater stingrays of the family Potamotrygonidae are highly exploited for the aquarium trade, management and conservation strategies need to be implemented at the level of each river basin, rather than at the level of the Amazon basin.

A família Potamotrygonidae forma um clado monofilético com três gêneros: *Paratrygon* Duméril, *Potamotrygon* Garman e *Plesiotrygon* Rosa, Castello & Thorson. A maioria das espécies dessa família possui distribuição restrita a uma única bacia ou sistema fluvial, e somente as espécies *Potamotrygon motoro*, *Potamotrygon orbignyi* e *Paratrygon aiereba* estão presentes em mais de uma bacia hidrográfica. O presente estudo teve como objetivo investigar a estrutura genética de *Paratrygon aiereba* em alguns rios da região Amazônica: Negro, sistema Solimões-Amazonas, Tapajós, Xingu, e Araguaia. Para tal foram utilizados como marcador molecular os genes de ATPase subunidade 6, e COI. As análises com o fragmento de COI indicaram que o gênero *Paratrygon* é grupo irmão dos outros gêneros da família potamotrygonidae. Os resultados para o fragmento de ATPase mostraram que essas populações estão estruturadas dentro dos rios, com fluxo gênico restrito, ou mesmo sem fluxo gênico, apresentando uma correlação positiva entre distância genética e distância geográfica. *Paratrygon aiereba* é composta por três clados com distância genética de pelo menos 2%. A divergência encontrada dentro desse grupo é semelhante à observada entre *Potamotrygon* spp. Segundo as premissas para barcoding COI e a distribuição alopátrica de três clados em *P. aiereba* indicam que esse grupo pode ser um complexo de espécies. O rio Negro é conhecido por sua pesca ornamental, e na calha Solimões-Amazonas, esses animais são utilizados como fonte de proteína e sofrem com a pesca comercial. Em vista disso medidas de conservação para esta espécie devem ser tomadas em nível local, considerando cada rio separadamente, ao invés de empregar escalas regionais maiores.

Key words: Amazon basin, ATPase, COI , Population genetics.

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Introduction

Phylogeography deals with principles and processes governing the geographic distributions of genealogical lineages (Avise, 2000). These analyses are aimed at investigating patterns of geographic distribution of taxa and understanding processes that have resulted in these patterns (Bermingham & Moritz, 1998). The Neotropical region has been the object of a large number of biogeographic studies on fishes. The complex geomorphological history of this region is reflected in its ichthyofauna, which provides a rich source of material for the study of the Neotropical biogeography (Vari & Malabarba, 1998). Many studies have been carried out with the aim of discovering these biogeographic patterns and interpreting these patterns in light of geomorphological processes such as the formation of hydrographic basins. Examples include studies of the genera *Brachyhypopomus*, *Pimelodella*, and *Roeboides* in Central America (Bermingham & Martin, 1998), fishes of the Neotropical family Rivulidae (Hrbek & Larson, 1999), *Potamorraphis* in the river basins of the Amazon and Orinoco (Lovejoy & de Araújo, 2000), *Prochilodus* in the Amazon, Orinoco, and Paraná River basins (Sivasundar *et al.*, 2001), *Leporinus* in the Paraná River Basin (Martins *et al.*, 2003), *Hypostomus* in the main river basins of the Neotropics (Montoya-Burgos, 2003), *Brachyplatystoma* in the Solimões-Amazon-Estuary system (Batista *et al.*, 2004; Batista & Alves-Gomes, 2006), *Arapaima gigas* in the Amazon River basin (Hrbek *et al.*, 2005), *Cichla* in the Orinoco and Amazon River basins (Willis *et al.*, 2007), *Symphysodon* in the Amazon River basin (Farias & Hrbek, 2008) and fishes of the order Characiformes in the main river basins of South America (Hubert & Renno, 2006).

The biotic diversification of aquatic systems is directly related to the geomorphological history of rivers basins. Inter-basin connections, alterations of drainage systems, and capture of rivers and basins promoted vicariance, and allopatric divergence. Thus, the geomorphological history of river and river basin formation provides a good model for studies of diversification of aquatic fauna (Bermingham & Moritz, 1998; Lundberg *et al.*, 1998; Montoya-Burgos, 2003).

According to Lundberg *et al.* (1998), a significant portion of the diversification of Neotropical freshwater fishes and other aquatic organisms occurred due to the formation of South American rivers and river basins at the end of the Cretaceous and the Cenozoic. One of the most important events in the diversification of ichthyofauna during this period involved marine incursions onto the continent. These marine incursions led to origin of freshwater fishes derived from marine groups such as anchovies, needlefish and freshwater stingrays (Lovejoy *et al.*, 1998).

Freshwater stingrays are endemic to the Neotropical region and belong to the family Potamotrygonidae (Carvalho *et al.*, 2003). This group is monophyletic and comprises three genera: *Paratrygon* Duméril (basal genus), *Potamotrygon* German and *Plesiotrygon* Rosa, Castello & Thorson (sister genera). These groups have 18 valid species, 15 of which are found in Brazil. Among these species, only *Potamotrygon motoro*, *Potamotrygon orbignyi*, and *Paratrygon aiereba* have wide distributions and are found in more than one hydrological basin. The group is found in diverse habitats, including beaches, flooded forests with a rocky, clay bottom and lakes (Carvalho *et al.*, 2003).

Although an exclusively freshwater group, these taxa share some biological characteristics with marine elasmobranches, such as low fecundity, late sexual maturation and slow growth rate (Araújo *et al.*, 2004). Such characteristics make the group vulnerable to commercial-scale exploitation. In the State of Amazonas, the family Potamotrygonidae is exploited by ornamental fisheries and is highly prized by the aquarium trade throughout the world. In the State of Pará, this family is also used as a food source, with the targeting of species that reach larger sizes, such as *Paratrygon aiereba* (Araújo *et al.*, 2001; Carvalho *et al.*, 2003).

Paratrygon aiereba is found in all types of aquatic environments of the Amazon basin (Carvalho *et al.*, 2003). This distribution is probably due the ability to adjust to the diverse ionic characteristics of Amazon River and its tributaries (Duncan *et al.*, 2009). The wide geographic range allied with low fecundity makes this species vulnerable to different environmental risks such as persecution, direct and indirect fisheries and habitat degradation (Araújo *et al.*, 2004; Martin, 2005). Because of their wide distribution and potentially vulnerable status, understanding how populations are structured has a high value for conservation.

The aims of the present study were to employ phylogeographic and population genetic analyses to obtain information on the distribution of genetic diversity of *Paratrygon aiereba*; to determine whether there is genetic structuring and whether such structuring is explained by river basins; and to discuss how these results can be used for the conservation of this organism.

Material and Methods

Tissue Sampling. Tissue samples were obtained from 65 specimens collected in several locations within the Amazon Basin. The collection sites (Fig. 1) included Solimões-Amazon-Estuary system (SAE, N=12), Negro River (NEG, N=16), Tapajós River (TAP, N=2), Xingu River (XIN, N=20), and Araguaia River (ARA, N=13). The tissue samples are in tisseu collection of Instituto Nacional de Pesquisas da Amazônia Inpa (Table 1).

DNA extraction and sequencing. Muscle tissue was collected and conserved in absolute alcohol and frozen at -20°C until DNA extraction. Tissue was dissolved using a SDS/proteinase K solution, and total genomic DNA was isolated using a standard phenol-chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989).

Two gene fragments of mitochondrial DNA were selected for the present study: cytochrome oxidase I (COI) and ATPase 6. Partial sequences from COI gene were used to

estimate genetic divergences, and to identify putative lineages or species within the *P. aiereba*, according to the DNA barcode guidelines. Other species of Potamotrygonidae and two outgroup species (*Himantura pacifica*, *Himantura schmardae*) available in the GenBank (data from Toffoli *et al.*, 2008) were also included in analyses.

The partial sequence of ATPase 6 was used for population genetic analyses, as well as to estimate intra-specific divergences and reconstruct phylogenetic hypothesis for the genus *Paratrygon*. Sequences of ATPase 6 of *Potamotrygon* and *Plesiotrygon* were used as outgroup.

Fragments of approximately 800 base pairs (bp) of the gene for ATPase 6 were amplified using the PotaATPf2_Lys (5'-GGGTCYAGCATTAGCCTTT-3') and PotaATPr2 primers (5'-GTTAGTGGTCAGGGGCTTGG-3') (Toffoli, 2006). DNA amplification via polymerase chain reaction (PCR) was performed with approximately 50 ng of total DNA, 10x buffer (100 mM of Tris–HCl, 500 mM of KCl, 15 mM of $MgCl_2$), 0.3 mM of dNTP (7.5µl), 0.3 mM of each primer (1.5µl), 3 mM of $MgCl_2$ (1.5µl), 1U of Taq Polymerase(1µl) and ddH_2O (8.5µl) for a final volume of 25 µl. The temperature profile was as follows: denaturation at 94ºC for 4 minutes, annealing at 50ºC for 1 minute, elongation at 72ºC for 1.5 minutes, repeated 35 times and a final elongation at 72ºC for 5 minutes.

The primers COIf.1 (5´-CTTAACACAACWTTCTTTGACCC-3´) and COIr.3 (5´-ACGTTTTGATGCRAAKGCYTCTC-3´) were kindly provided by Tomas Hrbek and used for the amplification of mitochondrial COI, using the same PCR conditions as for ATPase, but changing the annealing temperature to 53ºC. These primers amplify the COI region spanned by the primers COIf and COIa reported in Palumbi (1996); this region corresponds to the 3' half of the COI gene, while the standard COI Barcoding region corresponds to the 5' half of the COI gene.

The BigDye Cycle Sequencing kit (Applied Biosystems) was used for the COI sequences and the DYEnamic ET Terminator kit (GE-Healthcare) was used for the ATPase 6 sequences, following the manufacturers' instructions. Sequencing was preformed on the ABI 3130 x l automatic sequencer (Applied Biosystems) and MEGAbase 1000 (GE-Healthcare).

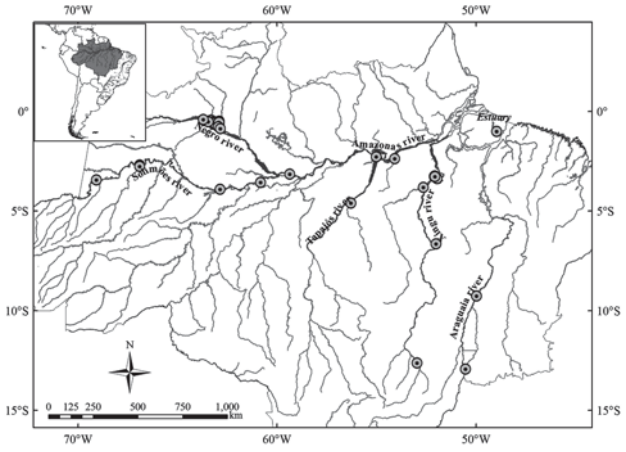

Fig 1. Map with the sample locations. In some localities more than one individual was sampled.

Data analysis. The sequences were manually edited and aligned with the aid of the BioEdit program (Hall, 1999). The most appropriate model of molecular evolution for each gene was selected using ModelTest (Posada & Crandall, 1998).

The haplotype tree phylogeny for COI sequences were constructed in Treefinder (Jobb *et al.*, 2004) under the Maximum Likelihood (ML) model of molecular evolution with parameters selected in the program ModelTest 3.7 (Posada, 2004), and neighbor-joining (NJ) method assuming the Kimura-two-parameter model of molecular evolution (barcoding standard - Hebert *et al.*, 2003). The NJ tree topology and statistical robustness (using 2000 bootstrap replicates) were estimated in PAUP* 4.0b10 (Swofford, 2002). Sequence data of other potamotrygonid species available in the GenBank (data from Toffoli *et al.*, 2008), were included in the analyses to calculate genetic divergence between genera and for the identification of putative lineages or species within the *P. aiereba*, according to the DNA barcode guidelines. The same two shark species used om Toffoli *et al.* (2008) were used as outgroups.

A haplotype tree of ATPase 6 was constructed in the program Treefinder (Jobb *et al.*, 2004) under the Maximum Likelihood (ML). The most appropriate molecular model was HKY+G as inferred in the program ModelTest 3.7 (Posada, 2004).

Based on the results obtained from the NJ and ML analyzes and delimiting clades by river systems, we subdivided the data into the following groups for further population-level analyses: the Solimões-Amazonas-Estuary system, Negro, Araguaia, Tocantins, and Xingu rivers. Due to the small number of specimens from the Tapajós River (N = 2), these individuals were excluded from the population level analyses. Molecular population level analyses such as nucleotide diversity (π), haplotype diversity (H), and number of polymorphic sites were performed using the program Arlequin 3.11 (Excoffier *et al.*, 2005). Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) neutrality tests were used to determine whether the samples from the different locations were at mutation-migration-drift equilibrium.

Population subdivision and structure were examined using analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) and the pair-wise Φ_{ST}, which is analogous to F_{ST} (Weir & Cockerham, 1984), both implemented in Arlequin 3.11 (Excoffier *et al.*, 2005). Significance of the correlation between the matrix of genetic distance and geographic distance between *P. aiereba* localities was tested by the Mantel test (Mantel, 1967) implemented in Arlequin 3.11 (Excoffier *et al.*, 2005). All tests that required multiple comparisons were corrected using the Bonferroni procedure (Rice, 1989).

Results

Cytochrome Oxidase I - COI. Considering that we were interested to check for inter and intra group divergences, we did not amplify all the individuals of *P. aiereba* for the COI gene.

Table 1. Vouchers of tissue sampling in Inpa collection.

DNA Collection INPA	Sample	Collection site	Latitude	Longitude
5220	SAE01	Solimões/Amazonas	-3.45	-69.06
5221	SAE02	Solimões/Amazonas	-2.70	-66.89
5222	SAE03	Solimões/Amazonas	-3.91	-62.85
5223	SAE04	Solimões/Amazonas	-3.58	-60.82
5224	SAE05	Solimões/Amazonas	-3.57	-60.82
5225	SAE06	Solimões/Amazonas	-3.15	-59.35
5226	SAE07	Solimões/Amazonas	-2.38	-54.08
5227	SAE08	Solimões/Amazonas	-2.76	-66.90
5228	NEG01	rio Negro	-0.71	-62.91
5229	NEG02	rio Negro	-0.46	-63.32
5232	NEG03	rio Negro	-0.54	-63.16
5238	NEG04	rio Negro	-0.46	-63.64
5241	NEG17	rio Negro	-0.46	-63.64
5243	NEG05	rio Negro	-0.64	-63.07
5244	NEG06	rio Negro	-0.64	-63.07
5247	NEG07	rio Negro	-0.59	-62.90
5251	NEG08	rio Negro	-0.53	-62.91
5253	NEG09	rio Negro	-0.53	-62.91
5254	NEG10	rio Negro	-0.78	-62.94
5255	NEG11	rio Negro	-0.78	-62.94
5262	NEG12	rio Negro	-0.88	-62.83
5281	NEG13	rio Negro	-0.528	-63.45
5289	NEG14	rio Negro	-0.46	-63.67
5304	NEG15	rio Negro	-0.46	-63.67
5305	NEG16	rio Negro	-0.46	-63.67
5307	ARA01	rio Araguaia	- 12.94	- 50.52
5308	ARA02	rio Araguaia	- 12.94	- 50.52
5309	ARA03	rio Araguaia	- 12.94	- 50.52
5310	ARA04	rio Araguaia	- 12.94	- 50.52
5311	ARA05	rio Araguaia	- 12.94	- 50.52
5312	ARA06	rio Araguaia	- 12.94	- 50.52
5313	ARA07	rio Araguaia	- 12.94	- 50.52
5314	ARA08	rio Araguaia	- 12.94	- 50.52
5315	ARA09	rio Araguaia	- 12.94	- 50.52
5320	ARA10	rio Araguaia	- 12.94	- 50.52
5322	ARA11	rio Araguaia	- 11.31	- 48.46
5324	ARA12	rio Araguaia	-9.27	-49.96
5325	ARA13	rio Araguaia	-9.27	-49.96
5326	XIN01	rio Xingu	- 6.66	- 52.00
5328	XIN02	rio Xingu	- 6.66	- 52.00
5329	XIN03	rio Xingu	-3.27	-52.08
5330	XIN21	rio Xingu	-3.37	-51.95
5331	XIN04	rio Xingu	-3.37	-51.94
5332	XIN05	rio Xingu	-3.38	-51.94
5333	XIN06	rio Xingu	-3.37	-51.95
5334	XIN07	rio Xingu	-3.27	-52.08
5335	XIN08	rio Xingu	-3.27	-52.08
5336	XIN09	rio Xingu	-3.27	-52.08
5338	XIN10	rio Xingu	-3.25	-52.08
5339	XIN11	rio Xingu	-3.25	-52.08
5340	XIN12	rio Xingu	-3.27	-52.08
5341	XIN13	rio Xingu	-3.27	-52.088
5342	XIN14	rio Xingu	-3.27	-52.09
5343	XIN15	rio Xingu	-3.27	-52.06
5344	XIN16	rio Xingu	-3.26	-52.05
5346	XIN17	rio Xingu	-3.82	-52.64
5348	XIN18	rio Xingu	-3.27	-52.06
5350	XIN19	rio Xingu	-3.27	-52.06
5353	SAE09	rio Amazonas	-2.28	-55.01
5354	SAE10	rio Amazonas	-2.28	-55.01
5355	TAP01	rio Tapajós	-4.61	-56.27
5356	TAP02	rio Tapajós	-4.61	-56.27
5380	ARA14	rio Araguaia	-12.63	-52.96
5381	SAE11	rio Arari	-1.01	-48.96
5382	SAE12	rio Arari	-1.01	-48.96

We randomly chose few individuals representative from each group. Among the 610 bp sequenced for the COI gene in 27 specimens of *P. aiereba*, 570 sites were monomorphic and 40 variable. The model that best explained the patterns of nucleotide substitutions found in the COI sequences was Hasegawa-Kishino-Yano. This model uses a variable nucleotide frequency and variable transition and transversion frequencies (Hasegawa *et al*., 1985), with the following parameters: TS/TV ratio = 7.7586; and nucleotide frequency of A=0.2775, T=0.2817, C=0.2753 and G=0.1655. The topologies and support values generated in the NJ (Fig. 2) and ML analyses (not shown) were very similar. The lineage formed by the genus *Paratrygon* was monophyletic and sister to the genera *Potamotrygon* and *Plesiotrygon.* The genetic distances between *Paratrygon* and the other genera and species of *Potamotrygon* based in Kimura-two-parameter model of evolution was very high, varying from 13% to 22% (Table 2). The inter-locality *Paratrygon* genetic distances varied from 0.37% (between SAE - NEG) to 4.9% (between SAE - ARA), while intra-locality genetic distances raged from 0.17% to 0.26%.

Within the genus *Paratrygon,* the group comprised of individuals collected from the Solimões-Amazon-Estuary system (SAE) and Negro River (NEG) formed a monophyletic group with a moderately high support value. The SAE+NEG clade was sister to individuals collected from Xingu River (XIN), with high support values. The clade formed by individuals collected from Araguaia River (ARA) was sister to the SAE+NEG+XIN clade (Fig. 2).

The genetic distances between *Paratrygon aireba* from the SAE system and NEG were low (Table 2). However, the divergence between XIN - SAE and XIN - NEG was 2.4% and 2.1%, respectively. The divergence between ARA - SAE and ARA - NEG was higher at 4.9% and 4.7%, respectively. Additionally, genetic distances within the genus *Paratrygon* were larger than those found among species of the rosette-spot group of *Potamotrygon* (*cf.* Toffoli *et al.*, 2008) comprises the following species: *Potamotrygon motoro*, *P. scobina*, *P. orbignyi*, *P. leopoldi*, *P. falkneri,* and *P. henlei*. Within *Paratrygon*, intra-clade divergences were less than 1%, as were the inter-specific divergences observed in the rosette-spot group of *Potamotrygon* (Table 2) (Toffoli *et al.*, 2008).

ATPase 6. Among the 584 bp sequenced for 63 individuals of *P. aiereba* considered for the analysis, 498 characters were constant and 86 were variable. The model that best explained the patterns of nucleotide substitutions found in the ATPase sequences was Hasegawa-Kishino-Yano+Gamma (HKY+G) with the following parameters: gamma=0.35, TS/TV ratio = 0.3006; and nucleotide frequency of A=0.30900, T=0.25140, C=0.34030 and G=0.09930. These parameters were used for the Maximum-Likelihood (ML) analysis (Fig. 3).

The haplotypes ML phylogenetic tree shows the same group observed in the COI tree, formed by SAE and NEG group, but with a low support value. Another group was formed by the TAP, XIN and ARA groups. A small group was formed by one individual from ARA and one from SAE showing no support values.

Population parameters for ATPase 6. The results of the DNA polymorphism are shown in Table 2. The greatest number of

Table 2. Genetic distance using COI sequence data based in Kimura-two-parameter model of evolution. Pair-wise comparison between *P. aiereba* and other species/group from data of Toffoli *et al*. (2008). Note: SAE= Solimões-Amazon River and Estuary, NEG= Negro River, XIN= Xingu River, ARA= Araguaia River.

Locality/Species	SAE	NEG	XIN	ARA	Rosette-spot group	*Plesiotrygon*	*P. schroederi*	Outgroup	Within Group
P. aiereba SAE	-								0.00259
P. aiereba NEG	0.00366	-							0.00167
P. aiereba XIN	0.02352	0.02057	-						0.00194
P. aiereba ARA	0.04941	0.04670	0.04215	-					0.00195
Rosette-spot group*	0.16950	0.16505	0.19420	0.17338	-				0.03037
*Plesiotrygon**	0.19809	0.19518	0.22070	0.19537	0.14631	-			-
*P. schroedery**	0.15918	0.15416	0.17282	0.14249	0.12658	0.15319	-		0.00398
Outgroup*	0.31106	0.30979	0.30689	0.30141	0.28962	0.37645	0.28980	-	0.04608

polymorphic sites (S) and greatest nucleotide diversity were found in the group from the Araguaia River. The greatest gene diversity values were found in the groups from the Solimões-Amazon-Estuary system, Negro, and Araguaia Rivers. Xingu River had the lowest DNA polymorphism values. Tajima's D was significant for the Solimões-Amazon system and Negro River populations, the latter of which also had significant Fu's Fs values (Table 3).

The AMOVA results revealed strong genetic structuring ($\Phi_{ST} = 0.71697$, $P < 0.001$), with 71.69% variation between rivers and 28.30% variation within rivers. Pairwise Φ_{ST} analyses also indicated strong differentiation among localities, and lack of exchange of individuals (Table 4).

Discussion

Barcoding. In order to facilitate a more rapid identification of species, the use of the barcoding, which promises the discrimination of species through the analysis of a small segment of the mitochondrial COI gene, is an innovative and efficient approach for the characterization of biological diversity (Hebert *et al.*, 2003). A growing number of studies have demonstrated that interspecific genetic distances observed between the COI mtDNA fragments is generally higher than 2%, which allows the differentiation of species that are more closely related and enables identification with a high degree of confidence (http://barcoding.si.edu).

The taxonomy and identification of species from the family Potamotrygonidae is difficult and new species are being described (Carvalho *et al.*, 2003; Carvalho & Lovejoy, 2011). The genus *Paratrygon* is monotypic (Rosa & Carvalho, 2007). However, due to its wide distribution, which ranges from the Orinoco River basin to the Amazon River basin, number of researchers have suggested that it a species complex (Charvett-Almeida, pers. comm.). The genetic analyses performed in this study are lending support to these conclusions. The COI fragment separated this species into three large groups: (1) the group from the Solimões-Amazon-Estuary system (SAE) and the Negro River (NEG); and (2) the group from the Xingu (XIN) River; (3) other group from Araguaia (ARA) River. The greatest genetic distance was observed between individuals from Araguaia River and the other localities with values above 4% of sequence divergence. The Xingu locality showed more than 2% sequence divergence from all other localities. These divergences are compatible with interspecific divergences under the molecular barcoding criteria (www.barcodinglife.org).

According to Moritz & Cicero (2004), a diagnosis with good accuracy depends on lower intra-specific variation in relation to inter-specific variation, the so called barcoding gap. Pairwise distances found between the SAE and NEG groups in relation to Xingu (~2%) and Araguaia (~4%) clades were roughly 10-fold greater than distances within each clade. According to Hebert *et al*. (2003), a threshold divergence 10-fold greater between clades than within each clade is normally found between different species. However, diagnosability of potamotrygonid species through DNA barcodes is not always efficient. Species of the *Potamotrygon* rosette-spot group composed of *Potamotrygon motoro*, *P. scobina*, *P. orbignyi*, *P. leopoldi*, *P. falkneri,* and *P. henlei*, share haplotypes and thus are not monophyletic (Toffoli *et al*., 2008). Haplotype sharing in this group could be due to incomplete lineage sorting in this recently diversified group, or due to introgressive hybridization. Intraspecific divergences are as large as interspecific divergences, and also exceed the suggested 2% divergence threshold (Toffoli *et al*., 2008), limiting the value of DNA barcoding for the *Potamotrygon* rosette-spot group.

Although barcoding has not functioned well for rosette-spot group of *Potamotrygon* (Toffoli *et al*., 2008), the identification system based on the COI fragment may be an initial step toward the delineation of species (Hebert *et al.*, 2003). In *P. aiereba,* the sister group of *Potamotrygon* + *Plesiotrygon*, COI has proven to be a good tool for the determination of evolutionarily significant units, and even potential new species, as the geographically restricted clades have lower within-clade genetic divergence than between clades, which is one of the premises of diagnostic accuracy based on the barcoding system (Hebert *et al*., 2003).

Biogeography. The results from the present study of the ATPase 6 reveal a separation into two large groups: one formed by the Solimões-Amazon-Estuary system and Negro River; and the other formed by the Tapajós, Xingu, and Araguaia Rivers, with the exclusion of the small group formed of one individual from SAE and one from ARA. These results match

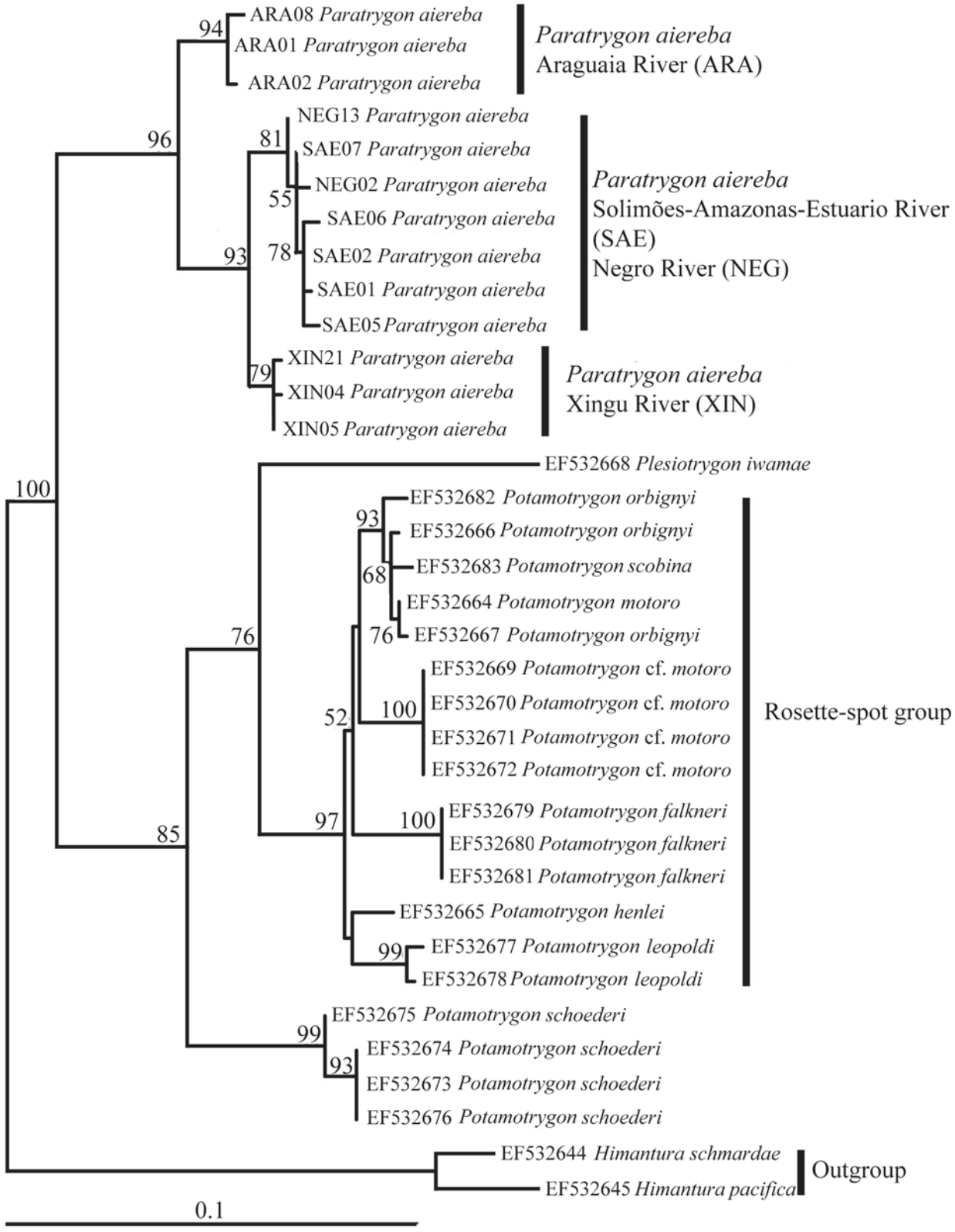

Fig 2. Neighbor Joining (NJ) tree for partial COI gene of *Paratrygon aiereba* combined with data from Toffoli *et al*. (2008). Numbers above the line are bootstrap support values.

Table 3. Population genetics parameters of ATPase 6 estimated for *Paratrygon aiereba*. SAE= Solimões-Amazon River and Estuary, NEG= Negro River, XIN= Xingu River, ARA= Araguaia River. * Level of significance P < 0.005; N = Number of individuals; S = Number of polymorphic sites; NH = Number of Haplotypes; H = Gene diversity; π = Nucleotide. * Level of significance P < 0.008, after Bonferroni correction.

Populations	N	S	NH	H	π	Tajima's D	Fu's *Fs*
SAE	12	21	7	0.8333±0.1002	0.0077±0.0046	-1.5456*	-0.2809
NEG	16	18	10	0.8917±0.0631	0.0053±0.0032	-1.6777*	-1.6777*
XIN	19	16	5	0.5906±0.1185	0.0096±0.0054	0.8618	43.536
ARA	14	63	8	0.7692±0.1198	0.0256±0.0137	-10.724	29.033
All	61	84	59	1.0000±0.0031	0.0349±0.0174	0.4445	-24.122*

the pattern of distribution found by Hubert & Renno (2006), who analyzed 345 species of Characiformes using parsimony analysis of endemicity and also found a separation into two large groups: (1) one denominated "Lower Amazon", corresponding to the Amazon, Negro, lower Madeira, and Branco Rivers; and (2) the other denominated "Xingu-Tocantins", corresponding to the Tocantins, Araguaia, Xingu, and Tapajós Rivers.

The Xingu, Tapajós, and Araguaia Rivers have their origin on the Central Brazilian Shield. The individuals collected from the Tapajós River, near the city of Itaituba, Pará State, and in the Xingu River, above Belo Monte, Pará, occur at the limit between the sedimentary basin and the Brazilian Shield, where large waterfalls are common, which may serve as a geographic barrier for this species, thereby playing an important role in the isolation of these populations.

The geomorphological events that resulted in the establishment of current river basins may have been agents of vicariant diversification, fragmenting and isolating these populations. Thus, one of the hypotheses that could explain the genetic pattern found in the present study is allopatric fragmentation and diversification of the *P. aiereba* populations. A number of biogeographic studies (Lovejoy *et al.*, 1998; Albert *et al.*, 2006; Hubert & Renno, 2006) have shown that the events of the Miocene period are the most important for the modeling of the distribution and evolution of Neotropical ichthyofauna.

According to Wesselingh & Salo (2006), the Amazon basin began to be delineated in their current configuration as a consequence of the emergence of the Andes, together with a variation in sea level due to the glaciers of the Quaternary, which carved out the rivers into what is more or less the current definition of the valleys. A number of authors agree with this formation process of the Amazon Basin, but the period in which it occurred is not yet well defined and ranges from 8 and 2.5 mya (Lundberg *et al.*, 1998; Campbell Jr. *et al.*, 2006; Wesselingh & Salo, 2006).

The family Potamotrygonidae is monophyletic, originating from a marine ancestor that colonized South America (Lovejoy *et al.*, 1998; Marques, 2000; Carvalho *et al.*, 2003; Carvalho *et al.*, 2004). The timing of the colonization remains tentative, ranging from 10 to 50 mya (Lovejoy *et al.*, 1998; Carvalho *et al.*, 2004). However, it uncontroversially precedes the final formation of the Amazon basin drainage system, and thus vicariance driven diversification of the potamotrygonid stingrays is a likely hypothesis.

Implications for conservation. As the genetic diversity of species represents the range of evolutionary and ecological adaptations in relation to a given environment, phylogeographic studies can generate useful information for the conservation of organisms (Avise, 2000). Based on the genetic divergence (see www.barcodinglife.org) of the populations from the Xingu, Araguaia, and the Solimões-Amazonas-Estuary system, the present study indicates that there is more than one species within what currently is considered *P. aiereba*. If these lineages represent different species, management and conservation policies will need to be modified to reflect this.

In the Brazilian State of Amazonas, freshwater rays are exploited by the ornamental fish trade for export to Europe, Japan, and the United States (Araújo *et al.*, 2004; Moreau & Coomes, 2007). In other regions of the Amazon, this group is also exploited as a food source throughout the Solimões-Amazon system, however in a small scale compared with the commercialization for the ornamental fish trade. Freshwater stingrays have also suffered from large-scale commercial fishing, in which they are caught as bycatch in gillnets (Araújo *et al.*, 2004).

In Brazil, at the end of the 1990s, the fishing of freshwater stingrays was banned by the Brazilian Environmental Agency (Instituto Brasileiro de Meio Ambiente e Recursos Naturais Renováveis - IBAMA (Araújo *et al.*, 2004). Since then, this activity has been permitted again and once again banned. Currently, the activity is permitted through the Normative Instruction nº 204/2008, which allows the capture of only six species from this family, with annual quotas, in the Amazon and Araguaia-Tocantins River basins in the states of Pará and Amazonas, (IBAMA, 2008). However, this norm is currently under review by IBAMA. *Paratrygon aiereba* is not among the six species that can be commercialized and its capture is prohibited. In Brazil, *P. aiereba* has a vulnerable status and it is prohibited to be exported as ornamental fish, but has been exported by Peru and Colombia in small quantities in the last fifteen years.

In other regions of South America, this species has been exploited by direct and indirect fisheries (Araújo *et al*., 2004; Barbarino & Lasso, 2005). However, other factors contribute to the vulnerability of this species: a low rate of population increment (low fertility) (Barbarino & Lasso, 2005; Charvet-Almeida *et al.*, 2005), low abundance rates (Almeida *et al.*, 2009); anthropogenic action in the environment (damns,

mines, dragging), persecution (Araújo *et al.*, 2004; Martin, 2005), and climate changes which affect the events of reproductive cycle. Further, with the enormous area to be covered, protective policies in the Amazon are very difficult to be implemented and reinforced. A large number of protected fish species, including the stingrays, are currently being unlawfully exported due to the lack of adequate infrastructure for fiscal policies in the region.

Using DNA barcoding one could identify putative illegal stingrays catches and exports. Although DNA barcoding cannot discriminate between the species of the *Potamotrygon* rosette-spot group, it can differentiate

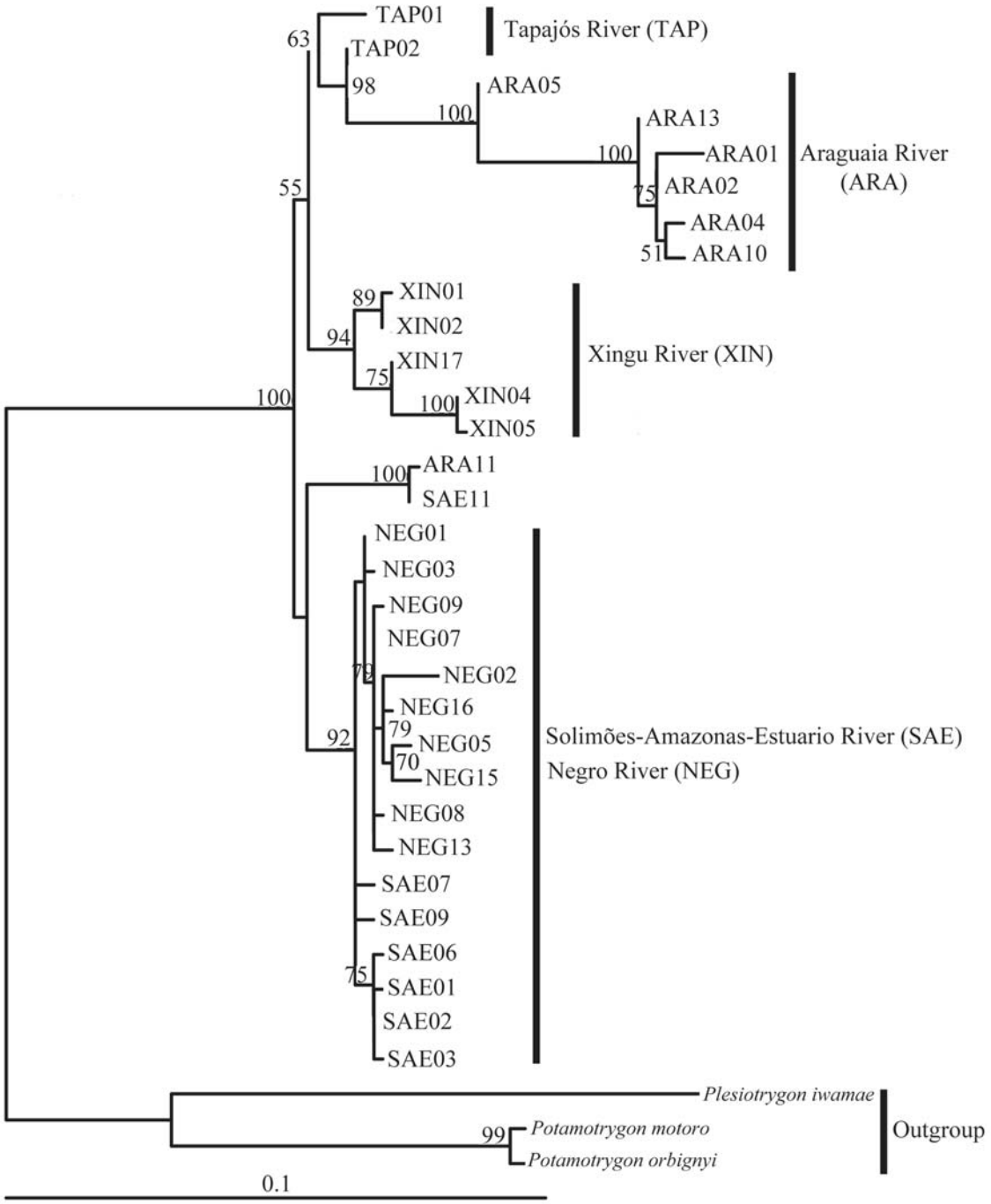

Fig 3. Maximum likelihood (ML) tree based on the analysis of the ATPase 6 gene of the *Paratrygon aiereba*. Numbers above the line are maximum likelihood edge support values.

Table 4. Indirect estimate of genetic differentiation (Φ_{ST}) (lower diagonal) and gene flow (Nm) (upper diagonal) of *Paratrygon aiereba*. SAE= Solimões-Amazon River and Estuary, NEG= Negro River, XIN= Xingu River, ARA= Araguaia River. * Level of significance P < 0.008; (after Bonferroni correction).

Populations	SAE	NEG	XIN	ARA
SAE	-	0.81261	0.23359	0.21522
NEG	0.38092*	-	0.15462	0.16978
XIN	0.68158*	0.76381*	-	0.18011
ARA	0.68158*	0.74651*	0.74651*	-

unambiguously between genera, as well as many species within the genus *Potamotrygon*. In the case of *Paratrygon aiereba* it can also differentiate between the geographic origins of the specimens. Considering that DNA barcoding is cheap and fast, it can be used to effectively implement conservation programs for freshwater stingrays by verifying that what is being exported or which areas are being fished, is in reality what is permitted to be sold.

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