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CELLULAR AND MOLECULAR BIOLOGY

Mitochondrial DNA suggests Hybridization in Freshwater Stingrays *Potamotrygon* (POTAMOTRYGONIDAE: MYLIOBATIFORMES) from the Xingu river, Amazonia and reveals speciation in *Paratrygon aireba*

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Abstract: In the Xingu river basin, Brazil, occurs two genera of Potamotrygonidae family: *Potamotrygon* and *Paratrygon*. In this region, the taxa have significant economic importance for the ornamental fishing industry, being intensively captured, especially the species *Potamotrygon leopoldi*, which is endemic to this basin. In the attempt to propose a species-specific DNA marker for the species from Xingu, as well as ensuring a robust and reliable molecular identification, the present work analysed mitochondrial gene portions Cytochrome Oxidase C – subunit I (COI) and Cytochrome B (Cytb) of five species: *P. leopoldi*; *Potamotrygon orbignyi*; *Potamotrygon motoro*; *Potamotrygon scobina* and *Paratrygon aiereba*. We found haplotype sharing, with a total absence of reciprocal monophyly in the majority of taxa. Individuals morphologically identified as a species showed mitochondrial DNA from another, suggesting the first record of hybridization amongst freshwater stingrays of Xingu. Also, we detected a deep divergence among *Paratrygon aiereba* haplotypes, indicative of speciation, suggesting the possibility of a new species for the Xingu river. Therefore, although the is still confusing and controversial taxonomy of freshwater stingrays, and evidencing hybridization processes that may have shaped the evolutionary history of this Family, the genes COI and Cytb can successfully help in the their species identification.

Key words: Potamotrygonidae, *Potamotrygon*, *Paratrygon aiereba*, Xingu river.

INTRODUCTION

The Potamotrygonidae family currently includes 32 species, organized into four genera: *Paratrygon* Duméril (1865), a monospecific genus; *Potamotrygon* Garman (1877), composed of 27 species, also the genera *Pleisiotrygon* Rosa, Castello & Thorson (1987) and *Heliotrygon* Carvalho & Lovejoy (2011) with two described species each (Fontenelle & Carvalho 2017). Recently Carvalho et al. (2016) proposed the inclusion of a new genus (*Styracura*) and subfamily (Styracurinae) in Potamotrygonidae, these taxa were initially placed in the marine stingray family Dasyatidae.

The freshwater stingrays have a restricted distribution to the South American rivers, which flow into the Caribbean and the Western Atlantic Ocean. Generally, they do not occur in the São Francisco river basin and coastal drainages from the east and south of Parnaíba river (Rosa et al. 2010). Some Potamotrygonidae species are

endemic to a single river basin, such as the White-blotched river stingray (*Potamotrygon leopoldi*), which is restricted to the Xingu river, in the Brazilian Amazon (Lasso et al. 2016, Rosa 1985, not published in indexed journals).

Additionally, some of the species have economic importance as ornamental fish in both national and international scenarios (Duncan et al. 2010). Ornamental fishing occurs extensively in the Xingu river basin. Thus, the intense capture of some taxa could compromise the sustainability of entire species populations, likewise impacting on the maintenance of this commercial activity in the region.

Furthermore, Potamotrygonidae is a group in which the taxonomy is still poorly understood. Recently it was subjected to many taxonomic revisions (Almeida et al. 2008, Loboda & Carvalho 2013, Lasso et al. 2016, Fontenelle & Carvalho 2017). The most extreme taxonomic revision was proposed by Carvalho et al. (2016), where it was proposed that a subdivision of Potamotrygonidae family be made into two subfamilies with the inclusion of marine species, previously placed in Dasyatidae family.

On the face of it, it is essential to use robust molecular tools capable of providing species-specific DNA marker which can be applied to the identification of freshwater stingrays. It can be the basis for conservation mitigation initiatives, contribute to export control, the sustainable maintenance of stocks, and effective acknowledgement of group diversity.

The fragment from the mitochondrial gene Cytochrome C oxidase subunit I (COI), an intraspecific conserved sequence that has the capability to show interspecific variations, was chosen to serve as standard DNA Barcode pattern, (Hebert et al. 2003, Ward et al. 2005, Pereira et al. 2013). Besides COI, Cytochrome b (Cytb) has been successfully applied in the identification of fish, showing as well an efficiency barcode marker (Chen et al. 2014, Palacios-Barreto et al. 2017). Moreover, its accentuated polymorphism can provide information about population structuring (Da Silva et al. 2015, 2016, 2018, Silva et al. 2018).

In the case of the neotropical freshwater ichthyofauna, many studies have confirmed the utility of COI as a Barcode method (Pereira et al. 2013). However in Potamotrygonidae, especially to the species from *Potamotrygon* genus, this marker did not show the ability to discriminate the taxa, particularly the species *P. motoro*, *P. scobina, P. orbiginyi* which constitutes the rosette-spot clade (Toffoli et al. 2008). The rosette-spot clade is a group of species that do not have reciprocal monophyly, which can be the result of recent phylogenetic radiation (Schluter 2000). Considering these pointed issues, followed by the dubiousness around the family taxonomy may collaborate as to the incapability of Barcode to separate the species (Toffoli et al. 2008). In addition to this, hybridization cases and/or incomplete lineages shared among Potamotrygonidae can also cover up the DNA Barcode efficiency (Pereira et al. 2013, Cruz et al. 2015).

There is not any genetic characterization found in the literature for the Potamotrygonidae species from the Xingu river basin. Even though the freshwater stingrays have a wide distribution over this basin, there is a record of an endemic species to the basin (*P. leopoldi*), as well as, the intense and deregulated exploitation of fish species for the ornamental fishing market. Consequently, many species are captured and commercialized without a secure record of what species are being taken, also, there is the possibility of hybrids individuals be compounding this commerce. This raises many concerns about the impacts of ornamental fishing and its sustainability, the impact on the maintenance of the overexploited stocks and

the threats to cryptic ichthyofauna yet to be described.

In this study, we employ molecular markers from the mitochondrial genome (Barcode fragments from COI and Cytb genes) for delimiting species of *Potamotrygon* and *Paratrygon* genera. We also, discuss the effectiveness of these mitogenomic regions as Barcode tool, and the possibility of speciation and hybridization in the Potamotrygonidae population along the Xingu river.

MATERIALS AND METHODS

Sampling

One hundred and sisty one samples were obtained for this study, which included the species: *Potamotrygon leopoldi* (102); *Potamotrygon orbignyi* (26); *Potamotrygon motoro* (11); *Potamotrygon scobina* (10); *Paratrygon aiereba* (4) and *Potamotrygon sp1* (4); *Potamotrygon sp2* (4). The sample collection was distributed along 20 localities over the main channel of the Xingu river basin, Brazil, as well as in their tributary's river, Bacajá river in the east margin and Iriri river in the west side (Figure 1).

The individuals were measured, photographed and identified checking the identification key species available for the Potamotrygonidae family (Loboda & Carvalho 2013, Fontenelle & Carvalho 2017). Tissue samples were collected from the pelvic fin, also, we verified if the individuals had already been sampled according to the presence of scars in the pelvic fins. All individuals were released back into their respective sampling locations. The tissue samples were preserved in cryogenic

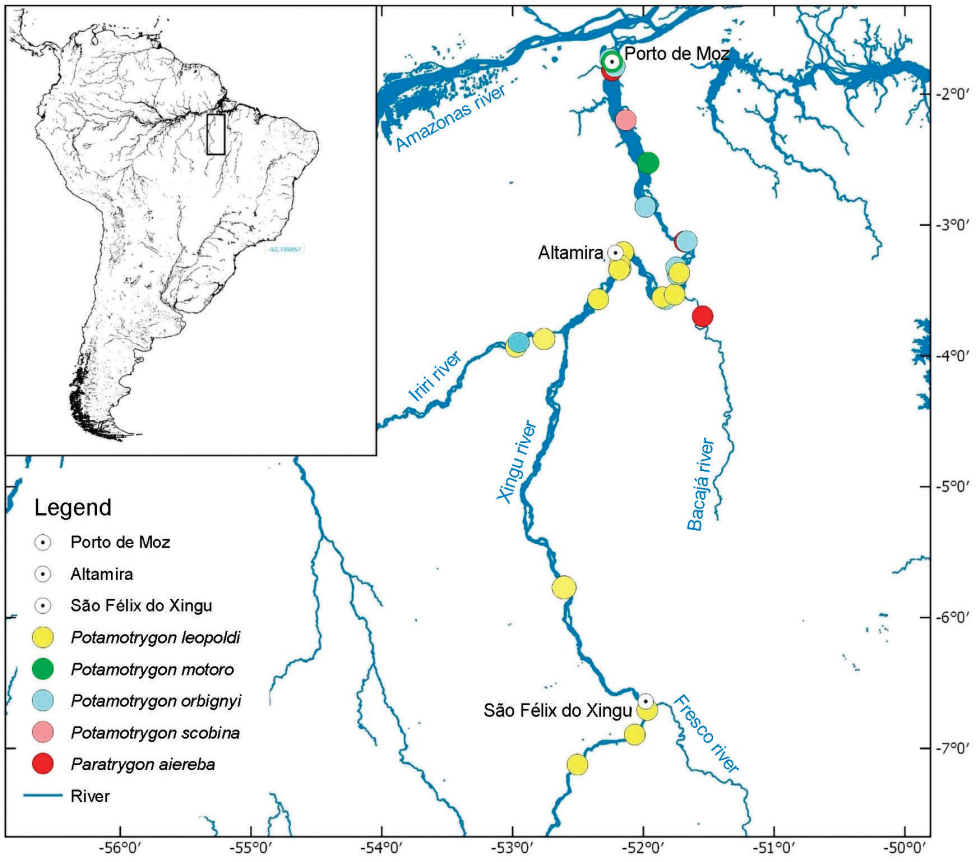

Figure 1. The study area map showing the collection sampling localities and where the respective species were found (*Potamotrygon leopoldi*=yellow; *Potamotrygon orbignyi*=blue; *Potamotrygon motoro*=green; *Potamotrygon scobina*=pink; *Paratrygon aiereba*=red).

tubes with ethyl alcohol, commercial (95%) and stored at -20 for posterior molecular procedures.

Laboratory procedures

Whole genome was isolated using the Wizard Genomic kit (Promega, Fitchburg, WI, USA), according to the manufacturer's instructions. Afterwards, the isolated DNA quality was verified by submarine electrophoresis for 40 minutes at 60V, in 1% agarose gel, with 2µL of a solution containing blue juice buffer and GelRed, visualized under UV light.

The Cytb fragment was isolated and amplified with the primes FishCytbF (ACCACCGTTGTTCAACTACAAGAAC) and TrueCytbR (CCGACTTCCGGATTACAAGACCG) (Sevilla et al. 2007) through the polymerase chain reaction (PCR). The final volume per reaction was 15µL, which included 2.5µL of dNTPs (1.25mM), 1.5µL of buffer solution (10x), 0.6µL of $MgCl_2$ (1.5mM), 0.6µL of each primer (50ng/µL), approximately 100ng (1µL) of template DNA, 0.1µL of Taq DNA polymerase (5U/µL), and ultrapure water to complete the reaction (8.1µL). The PCR conditions were carried out as follows: a first denaturation at 95°C for 4s, 35 cycles of denaturation at 95°C for 30s, hybridization at 54°C for 40s and extension at 72°C for 90s, added by a final extension at 72°C for 5 min.

The COI Barcode region was amplified with the primers FishF1 (TCAACCAACCACAAAGACATTGGCAC) and FishR1 (TAGACTTCTGGGTGGCCAAAGAATCA) (Ward et al. 2005), succeeding the same PCR conditions described to Cytb. The COI amplification cycles comprised: a first denaturation at 95°C for 3s, 35 cycles of denaturation at 95°C for 30s, hybridization at 56°C for 45s and extension at 72°C for 60s, added by a final extension at 72°C for 3 min.

The successful PCR reactions products were purified with PEG (polyethylene glycol), according to Paithankar & Prasad (1991) and sequenced using the dideoxy method (Sanger et al. 1977), with the Big Dye kit (ABI Prism™ Dye Terminator Cycle Sequencing Reading Reaction – PE Thermo Fisher). The precipitated product was submitted to capillary electrophoresis in ABI 3500XL automatic sequencer (Thermo Fisher).

Dataset

We constructed individual datasets for each molecular marker. The sequences obtained were edited and aligned in BioEdit software v. 7.1.3.0 (Hall 1999), and the automatic alignment was carried out in CLUSTALW (Thompson et al. 1994), implemented using the same software. The polymorphic sites and possible species-specific mutations were visualized in MEGA v 7.0 (Kumar et al. 2016), as well as stop codons. The mtDNA haplotypes characterizations (number identification and frequency) were determined in the DnaSP software v 5.10 (Librado & Rozas 2009).

Species Identification and Phylogenetic inferences

In MEGA v7.0 software (Kumar et al. 2016), the neighbour-joining tree (NJ) was constructed under the Kimura-2-parameter (K2p) (Kimura 1980), for each marker. This was also used to direct the sample subdivisions into groups/taxa and for the corrected genetic distance calculation (K2P model). The statistical support for the tree branches was obtained through 1.000 bootstrap pseudoreplicates. To identify the presence of Barcode gap, we calculated the interspecific and intraspecific mean distance.

Bayesian-inference trees (BI) was carried out using BEAST software v. 1.10.4 (Drummond & Rambaut 2007, Drummond et al. 2012). The best tree was read on R software with the Splits package. For all markers, we used the uncorrelated lognormal relaxed clock. The Yule process was used as prior for the species tree.

We also performed an independent chain of 5 x 10^7 with sampling parameters each 5.000 generations. The log files were inspected on Tracer v1.5 (Rambaut & Drummond 2012) to evaluate the convergence chain and the adequate mixing and burn-in length.

The maximum likelihood topology (ML) (Pons et al. 2006) for the examined genes was constructed in the RAxML v.8.29 software (Stamatakis 2014) and 1.000 bootstrap pseudoreplicates. In jModelTest 0.1.10 software (Darriba et al. 2012, Posada 2008), the nucleotide substitution models which better adapted to the dataset were chosen using the Akaike information criterion (AIC), considering each marker. The following models were chosen: HKY+I (Hasegawa & Yano 1984) for COI and TrN+I (Tamura & Nei 1993) for Cytb.

As outgroup, for the Cytb analysis, sequences from GenBank from the species *Himantura pacífica* (AF110638; Lovejoy et al. 1998) and *Himantura chophraya* (KX668133; Khudamrongsawat et al. 2017) were included in the dataset. These species compounds the most likely sister taxa (Dasyatidae family) of Potamotrygonidae family (Dunn et al. 2003, Lovejoy 1996, Lovejoy et al. 1998). Conjointly, other sequences of freshwater stingrays from different river basins were added to the analysis, *Potamotrygon motoro* (JN020040; JN020041; JN020044), *P. orbignyi* (AF110625; Lovejoy et al. 1998) e *Paratrygon aiereba* (AF110629; Lovejoy et al. 1998). In the case of COI analysis, the same approach was adopted, being the species *Himantura uarnak* chosen as outgroup (NC028325; Shen et al. 2016), and *Potamotrygon motoro* (JN989157; JN989160), *P. falkneri* (JN989145) (Pereira et al. 2013) and *Paratrygon aiereba* (KX688093; Kirchhoff et al. 2014) from distinct river basins.

Species delimitation

Different species delimitation approaches were employed considering methods based on trees and the coalescent theory or genetic distance, as described below:

Automatic Barcode Gap Discovery (ABGD): We used the web version of ABGD, available at < http://www.abi.snv.jussieu.fr/public/abgd/abgdweb.html>. It is a method based on the differences between species pairs to establish the barcode gap and is a highly sensitive method according to the provided similarity threshold. Because of that, we applied the Pmax values (0.1). All analysis was set using simple distance (p). We used two relative gap width values (X=0.2), and the rest remained as default. For the results, we considered the four first partitions generated by the software.

Generalized Mixed Yule-Coalescent (GMYC): The GMYC requires as input an ultrametric tree. We adopted the model "single threshold" (-log ML = 434.24) which was better suited to our data. In addition, we used a gene tree estimated on BEAST v. 1.10.4 (Drummond & Rambaut 2007, Drummond et al. 2012), performed in 50 million generations and a sampled tree inspected each 5.000 generations accepting the substitution model TrN+I in the lognormal relaxed clock model (uncorrelated). With the Yule process approach was inferred the node trees age analysis. The chain convergence was evaluated on Tracer v1.6 by either graphic inspection or ESS values (Effective Sampling Size). Adequate considered convergence chains were higher than 2500 ESS. Lastly, the sampled trees were summarized on TreeAnotator v1.8.0 software using a bur-in of 2500. Subsequently, the GMYC analysis was run in the implemented gmyc function in R software with the packet splits (Ezard et al. 2014). The analysis was achieved through testing of single and multiple thresholds.

Poisson Tree Process (PTP): A gene tree for each gene (COI and Cytb) was applied as input using the maximum likelihood method in RAxMl software v.8.29 (Stamatakis 2014). A simple genetic distance tree was utilized as input (p), which were estimated by the NJ approach on MEGA v7.0 (Kumar et al. 2016). The runs were carried out through the web server bPTP, available at <http://species.h-its.org/ptp/>. The analysis was submitted as the software´s default.

RESULTS

Databases and haplotype frequency

Considering the five species of this study, our dataset comprised 116 sequences of Cytb (MZ328336 - MZ328451) and 117 sequences of COI (MZ321865 - MZ321981). The length after the alignment and edition were 670bp for Cytb, which where were observed the presence of 81 polymorphic sites. The COI dataset length had 500 bp, containing 75 polymorphic sites. Respectively the markers did not present stop codons. The Tables I and II shows the list of haplotypes for each species, the number of

Table I. Haplotype list for Cytochrome B (Cytb), the frequency in each species, the number of individuals sequenced in each species for each marker, along with the haplotype number observed by species (showed in parentheses). The species which shared the same haplotype are highlighted (in grey).

Cyt b Haplotypes	*Potamotrygon motoro*	*Potamotrygon scobina*	*Potamotrygon orbignyi*	*Potamotrygon leopoldi*	*Potamotrygon* sp.1	*Potamotrygon* sp.2	*Paratrygon aiereba*
Hap-1	-	-	-	19	-	1	-
Hap-2	-	-	-	14	-	-	-
Hap-3	-	-	-	8	-	-	-
Hap-4	-	-	-	7	-	-	-
Hap-5	-	-	-	16	-	1	-
Hap-6	-	-	-	2	-	-	-
Hap-7	-	-	-	1	-	1	-
Hap-8	5	-	-	-	-	-	-
Hap-9	2	-	-	-	2	-	-
Hap-10	3	-	-	-	1	-	-
Hap-11	1	-	-	-	-	-	-
Hap-12	-	5	6	-	-	-	-
Hap-13	-	1	-	-	-	-	-
Hap-14	-	1	-	-	-	-	-
Hap-15	-	-	-	-	-	-	2
Hap-16	-	-	-	-	-	-	1
Hap-17	-	-	1	-	-	-	-
Hap-18	-	-	2	-	-	-	-
Hap-19	-	-	5	-	-	-	-
Hap-20	-	-	1	-	-	-	-
Hap-21	-	-	1	-	-	-	-
Hap-22	-	-	4	-	-	-	-
Hap-23	-	-	1	-	-	-	-
Hap-24	-	-	1	-	-	-	-
TOTAL	**11 (4)**	**7(3)**	**22 (9)**	**67 (7)**	**3 (2)**	**3 (3)**	**3 (2)**

sequenced individuals by marker, as well as, the total haplotype number by species and species identification based on morphological characters.

In the 116 sequenced individuals for Cytb, 24 haplotypes (Hap) were found, being the most frequently shared between 19 *P. leopoldi* individuals. *Potamotrygon orbignyi* was the species which presented higher haplotype variations, presenting nine in total. The Hap 12 was the most frequent in this species, it is also shared conjointly with *P. scobina* individuals (Table I).

The 117 COI sequences comprised 18 haplotypes overall. The *P. leopoldi* species presented the higher number of Hap variations, consisting in general eight haplotypes. Haplotype 2 was the most frequent, being shared among 38 individuals, followed by Hap 1 which were detected in 19 individuals. Others showed Hap frequencies of 12 or below were exclusive specimen's. The Haplotype 9 was shared among 11 individuals, including *P. motoro*, *P. orbignyi* and *Potamotrygon sp1.* specimens. Similarly, the same pattern of sharing was found for the Hap 11, which could be detected among *P. scobina* and *P. orbignyi* individuals (Table II).

Molecular identification and species delimitation

The Neighbour-joining (NJ) trees generated very similar topologies, as shown in Figures 2 and 3. The arrangements, in some cases, clustered the species into different groups in contrast to previous morphological identification. Thus, different species that were placed in the same group or haplotypes of a single species were shared among different groups, as noticed with *P.*

Table II. Haplotype list for Cytochrome Oxidase C – subunit I (COI), the frequency in each species, the number of individuals sequenced in each species for each marker, along with the haplotype number observed by species (showed in parentheses). The species which shared the same haplotype are highlighted (in grey).

COI Haplotypes	*Potamotrygon motoro*	*Potamotrygon scobina*	*Potamotrygon orbignyi*	*Potamotrygon leopoldi*	*Potamotrygon* sp.1	*Potamotrygon* sp.2	*Paratrygon aiereba*
Hap-1	-	-	-	19	-	2	-
Hap-2	-	-	-	38	-	1	-
Hap-3	-	-	-	2	-	-	-
Hap-4	-	-	-	1	-	-	-
Hap-5	-	-	-	1	-	-	-
Hap-6	-	-	-	2	-	1	-
Hap-7	-	-	-	2	-	-	-
Hap-8	-	-	-	3	-	-	-
Hap-9	8	-	1	-	2	-	-
Hap-10	3	-	1	-	1	-	-
Hap-11	-	5	6	-	-	-	-
Hap-12	-	1	-	-	-	-	-
Hap-13	-	-	-	-	-	-	1
Hap-14	-	-	-	-	-	-	1
Hap-15	-	-	5	-	-	-	-
Hap-16	-	-	8	-	-	-	-
Hap-17	-	-	1	-	-	-	-
Hap-18	-	-	1	-	-	-	-
TOTAL	**11 (2)**	**6 (2)**	**23 (7)**	**68 (8)**	**3 (2)**	**4 (3)**	**2 (2)**

orbignyi and *P. scobina*. In the NJ arrangements, Potamotrygonidae genera were always kept separated from the outgroup and apart from themselves ingroup. The *Potamotrygon* genus species were grouped into an only clade, strongly well supported, separated from *Paratrygon*. The *Paratrygon* genus presented diverging haplotypes even though it is taxonomically monotype genus. In general, there was no presence of species-specific groups, with exception to *Paratrygon aiereba*.

In haplotype tree, the observed groups for both COI and Cytb markers were mostly concordant. Considering the NJ topology for Cytb fragment (Figure 2), it clustered the species into two major groups. The first comprised *P. leopoldi*, *P. orbignyi* and *P. scobina* species, along with individuals identified as *Potamotrygon* sp2., which represented 14 haplotypes with a mean

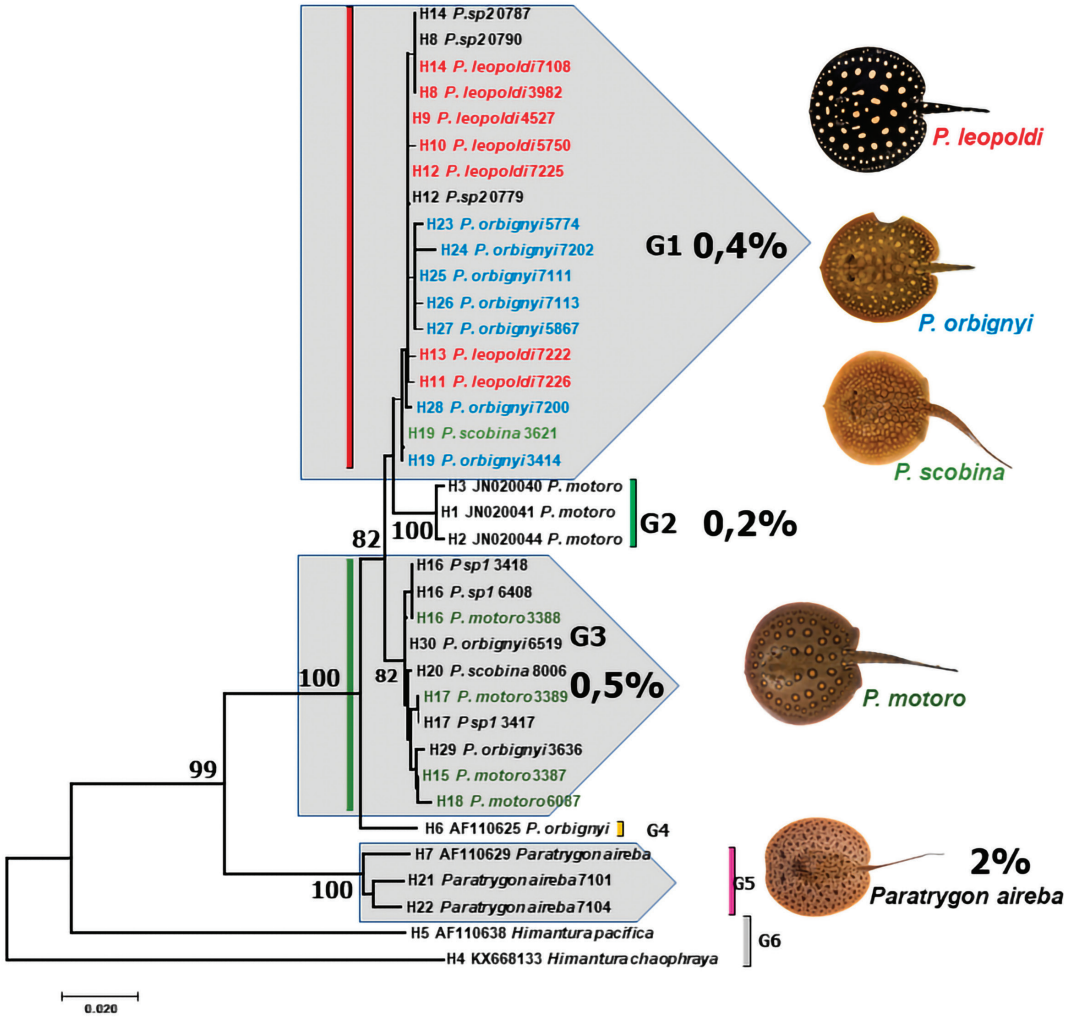

Figure 2. Neighbour-joining tree (NJ) from the haplotypes (H) of the mitochondrial gene Cytb of the Potamotrygonidae from the Xingu river basin: genera *Potamotrygon* and *Paratrygon*. Percentages in the groups' nodes (G) refers to the mean intrapopulational distance. Individuals of the species with different colors in the clades: *Potamotrygon leopoldi* in red, *Potamotrygon orbignyi* in blue, *Potamotrygon scobina* in green, *Potamotrygon motoro* in dark green and *Paratrygon aireba* in black. (Stingray' source of images: Leandro Sousa).

intraspecific distance of 0.4%. The second group joined only the three haplotypes of *P. motoro* taken from the public database of nucleotide sequences (GenBank), which they did not group with individuals identified as *P. motoro* from Xingu river. In contrast, these specimens were clustered in a third clade, simultaneously with *P. orbignyi*, *P. scobina* and *P. sp1*, having an intraspecific mean distance of 0.5%. The fourth group comprised haplotypes of *P. orbignyi* obtained from GenBank. The five groups were constituted of haplotypes of *Paratrygon aiereba* species, including individuals from GenBank, this clade group had a mean divergence of 2%. Lastly, species of *Himantura* genus were used to root the tree, categorized as group six. In the case of COI marker, presented concordant arrangements, except for individuals of *P. scobina*, which were separated from *P. leopoldi* and *P. motoro* species, which clustered together *P. orbignyi*, therefore sharing haplotypes (Figure 3).

This combination of individuals from distinct species was noticed in the results of different

Figure 3. Neighbour-joining tree (NJ) from the haplotypes (H) of the mitochondrial gene COI of the Potamotrygonidae from the Xingu river basin: genera *Potamotrygon* and *Paratrygon*. Individuals of the species with different colors in the clades: *Potamotrygon leopoldi* in red, *Potamotrygon orbignyi* in blue, *Potamotrygon scobina* in green, *Potamotrygon motoro* in dark green and *Paratrygon aireba* in black.

tests of species delimitation (Figures 4 and 5). The ABGD analysis using Pmax = 0.20 and Pmax = 0.10, had similar results when the relative gap extent (X) was equal to 2.0. In both cases, COI (Figure 4) and Cytb (Figure 5), the analysis resulted in four partitions and four groups. One group were constituted of *P. motoro-P. scobina-P. orbignyi*, another of *P. leopoldi-P. scobina-P. orbignyi*. These group individuals did not match the morphological identification for its species. Only

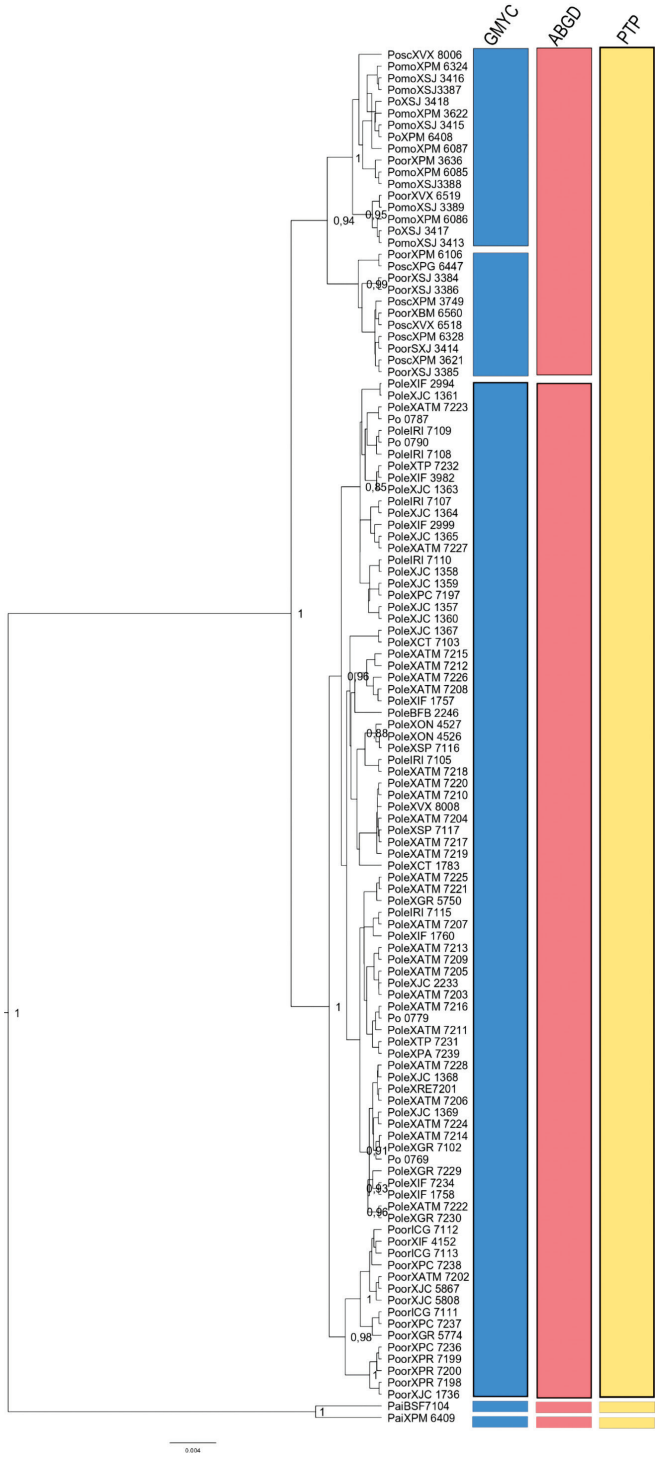

Figure 4. The ultrametric tree inferred in BEAST based on the COI gene, also featuring the species delimitations for this marker. It is showing the results of ABGD analysis taking into account the first four partitions (pink bar). In addition the GMYC delimitation results, adopting the Yule process (blue bar) and PTP (yellow bar) are shown. Along with, the posterior probabilities values above 0.8 from the Bayesian tree. Species Codes: Posc - *Potamotrygon scobina*; Pomo - *Potamotrygon motoro*; Poor - *Potamotrygon orbignyi*; Pole - *Potamotrygon leopoldi*.

the monotype genus *Paratrygon* was subdivided into two distinct groups, corroborating with the NJ topology.

Concerning the GMYC analysis, the results were different for the two mitochondrial markers (Cytb and COI). Through the use of the Yule process in the Cytb region, the species delimitation resulted in four independent coalescent groups: (1) *P. motoro-P. scobina-P. orbignyi* (2) *P. leopoldi-P.scobina-P. orbignyi*, and two distinct groups of *P. aiereba* (Figure 4). The same species delimitation to the COI region resulted in five groups: *P. motoro- P. scobina-P. orbignyi*; (2) *P. scobina-P. orbignyi*, (3) *P. leopoldi-P. orbignyi*; (4) *P. aiereba*; (5) *P. aiereba* (Figure 4). The COI results did not correspond to the groups defined morphologically and they divided the monotype genus *Paratrygon*.

In the case of PTP analysis, there were no differences in the analyses for the molecular markers COI and Cytb. Both results point out the existence of three OTUs, which did not separate the morphologically classified species, clustering *P. motoro-P. orbignyi- P. scobina-P. leopoldi* in a single group and *Paratrygon* divided into two groups (Figures 4 and 5).

Genetic divergence

In the evaluation of the genetic divergence among the five species analysed, considering the Cytb fragment, we found interspecific values in the genus *Potamotrygon* varying over 0.6%. Furthermore, among the groups: *P. leopoldi-P. orbignyi*, *P. motoro-P. leopoldi* and *P. motoro-P. orbignyi* 1.5% respectively. In the case of genus *Paratrygon* and the species of *Potamotrygon*, the distances levels varied from 9.2% to 9.6%.

To COI, the interspecific mean distance in *Potamotrygon* genus varied from 0.7% in the comparisons between *P. motoro-P. scobina*, and between *P. motoro-P. orbignyi* 2.2%. The genetic distances between *Paratrygon* and other *Potamotrygon* genus species varied from 12.7% to 13.9%. The divergence among the haplotypes of *P. motoro* from Xingu river and *P. motoro* Paraná river (sequences available on GenBank) presented values higher than 2%.

Considering the groups observed in the NJ tree, all the species used as the outgroup were remarkably divergent from the Potamotrygonidae analysed. They presented differentiations higher than 19% in all comparisons and molecular markers employed. The *Potamotrygon* groups showed divergences less than 3% in many comparisons. In the case of the groups *P. leopoldi-P. orbignyi* and *P. motoro*/Xingu-*P. scobina-P. orbignyi*, the divergences were less than 2%, thus showing no clear interspecific boundary of genetic divergence.

In the intraspecific distances to Cytb, the values of *P. motoro* were less or equal of 0.3%, for *P. leopoldi* were 0.2%, *P. scobina* 0.5%, *P. orbignyi* 0.6% and *Paratrygon aiereba* 1.5%. Concerning the COI, all the species analysed had values below to 0.4%, in exception for specimens of *P. aiereba*, which had divergences over 1%. In the case of ingroups which the species shares the same haplotypes, all the intraspecific distances were less than 0.5%.

Phylogenetic inferences

Both analyses (ML and BI) had highly congruent results, generating analogous arrangements, thus, we choose to show only the BI tree. The Potamotrygon group of species showed monophyletic arrangement with high support (BI=0.98), though the arrangements over species were not clear. Both trees had strong support between the genera *Paratrygon* and *Potamotrygon*. *Paratrygon* lineages presented monophyletic and as the sister group of *Potamotrygon* genus (Figures 4 and 5).

Potamotrygon orbignyi individuals collected in the upstream rapids of Xingu

river have haplotypes more closely related to *P. leopoldi* species than individuals from downstream Xingu. Thus, *P. orbignyi* individuals from the upstream occur in sympatry with *P. leopoldi*. While individuals of *P. orbignyi* and *P. scobina* collected on downstream Xingu shared haplotypes with *P. motoro*, taking account COI marker, likewise for Cytb with *P. leopoldi*. In other

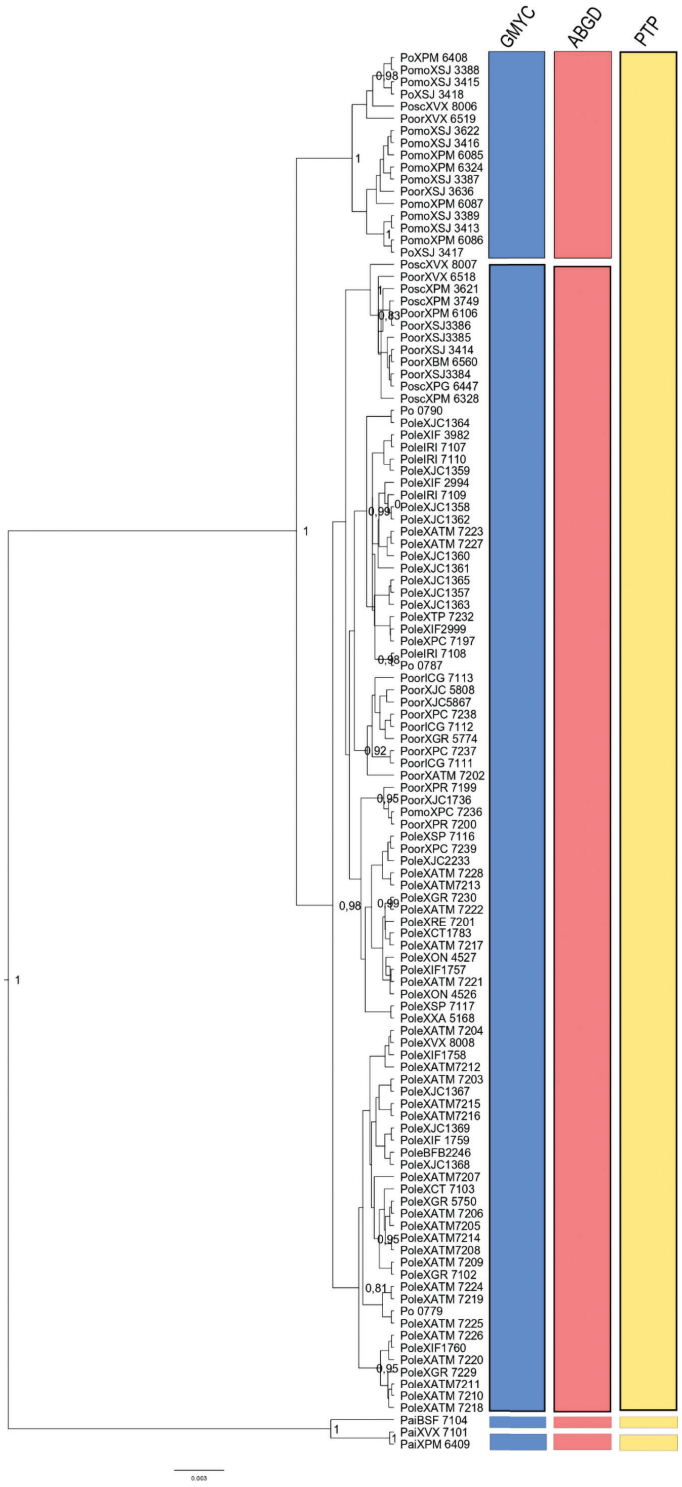

Figure 5. The ultrametric tree inferred in BEAST based on the Cytb gene, also featuring the species delimitations for this marker. It is showing the results of ABGD analysis taking into account the first four partitions (pink bar). In addition the GMYC delimitation results, adopting the Yule process (blue bar), and PTP (yellow bar) are shown. Along with, the posterior probabilities values above 0.8 from the Bayesian tree. Species Codes: Posc - *Potamotrygon scobina*; Pomo - *Potamotrygon motoro*; Poor - *Potamotrygon orbignyi*; Pole - *Potamotrygon leopoldi*.

words, the couple of molecular markers suggest two major groups for *P. orbignyi*, being one from Middle Xingu, associated with *P. leopoldi*, and another group from downstream Xingu, which shares haplotypes with *P. scobina*.

In all analysed trees, the branches which contained *Paratrygon* specimens presented high support (BI=1), forming distinct branches for those individuals collected in the main Xingu river channel, also another branch which comprehends the specimen collected in the middle Xingu´s river left tributary, the Bacajá river. This result may indicate the possibility of this individuals be a new species for *Paratrygon* genus.

DISCUSSION

Molecular identification and hybridization evidence

According to morphological identifications, five species were analysed in the present study, which consisted of two genera: *P. motoro*, *P. orbignyi*, *P. scobina*, *P. leopoldi* and *Paratrygon aiereba*. The use of mitochondrial markers for molecular identification was not successful in identifying a clear separation among the taxa under analysis. This was especially the case for *Potamotrygon* genus, which showed an absence of reciprocal monophyly among the lineages. As constantly observed in our analysis, different species from *Potamotrygon* genus shared the same mitochondrial haplotypes.

Even though the markers efficiently separated the different genera, they showed a subdivision in the monotype genus *Paratrygon*. Furthermore, specimens where previous morphological identification was not accurate could be clustered by the respective species with the use of molecular tools. They classified *Potamotrygon sp.1* as *P. motoro* and *Potamotrygon sp.2* as *P. leopoldi*.

The mitochondrial haplotypes sharing between *P. orbignyi* and *P. scobina* may have different causes. Mostly these can be related to morphological misidentification of specimens or associated with a hybridization scenario between these species. On one occasion, the individuals have an uniparental mitochondrial inheritance from one species and display morphological characteristics from another species. However, this may not necessarily exclude the efficiency potential of these markers as Barcode tools.

This pattern of extensive mitochondrial haplotypes sharing among freshwater stingrays species (*P. motoro, P. scobina and P. orbignyi*) has already been reported in the work of Toffoli et al. (2008). According to the authors, it is possible that this occurring incomplete lineages sorting is due to recent speciation events in these taxa, which results in the limitation of using the Barcode for the identification of new species. Therefore, beyond considering DNA sequences, it is necessary to carry out an integrative taxonomy, considering morphological data, ecological aspects, and therefore, consequently defining a taxonomy for this taxon (Toffoli et al. 2008).

Hybridization events already have been reported in this group in other hydrographic basins. As in the Paraná river case, where individuals showing morphology type of *P. motoro* shared haplotypes of COI and Cytb genes with *P. falkneri*. Latterly, it was confirmed with the use of microsatellites data (Cruz et al. 2015), as well as, multiple introgression events and introgressive hybridization were found.

The non-reciprocal monophyly of species observed in the trees is corroborated in the different delimitation tests, as conjointly demonstrated by Toffoli et al. (2008). In the ABGD approach, there were no significant differences over the regions used (Cytb and COI), with both defining four major groups. The method

clustered the four morphologically defined *Potamotrygon* species into two groups, whereas the monotype genus *Paratrygon* was subdivided into two groups, similarly to what was found in the GMYC analysis.

There were no differences in the mitochondrial regions analysed adopting the PTP approach. Both surveys defined three species hypotheses, so it does not agree with the five morphologically identified species under analysis. Although the confusion we found to delimit the species of *Potamotrygon* genus, all methods strongly indicate that the monotype *Paratrygon* genera are subdivided, suggesting speciation in the individuals from Xingu river basin.

The GMYC methodology demonstrated to be more effective in the attempt to separate the freshwater stingray species, likewise other fish groups (Tang et al. 2014, da Silva et al. 2018). The ABGD and PTP were not capable of distinguishing many species that are easily recognized by morphological traits, as *P. leopoldi* for instance, which has a singular morphology over other Potamotrygonidae. The lowest number of species grouped was defined in the PTP method, which in other studies, showed the same pattern of results in comparison to other applied methods (Tang et al. 2014, da Silva et al. 2018).

The apparent lack of resolution using mitochondrial markers to identify Potamotrygonidae, is possibly related to recent hybridization events which occurred in the Xingu river basin. However, the molecular tools were very efficient in separating the analysed genera, including indicating a new species of freshwater stingray to the region.

Due to the presence of hybrids, followed by the evolutionary history of the group, which suggests recent speciation events, it was not possible to define a Barcode gap to the mitochondrial regions utilized. The exceptions were the two genera of Potamotrygonidae, and the lineages inside *Paratrygon*, where the biggest levels of intrapopulation genetic divergence were lower than the lowest values of interspecific divergence.

The genetic distance of *Paratrygon* clade concerning other species of *Potamotrygon* varied from 12.7% to 13.9%. This corroborates with findings of a study of *Paratrygon aiereba* (Frederico et al. 2012), where divergences between two lineages were observed in this species, presenting values bigger than the registered in relation with species of *Potamotrygon* genus (2.4%).

This highlights that it is only possible to use mitochondrial regions as Barcode marker to identify species of fish with well-defined taxonomy, disregarding hybridization cases when taking account single locus analysis, including batoids species (Toffoli et al. 2008, Cerutti-Pereyra et al. 2012).

Although the Cytb showed to have the most haplotype variability (Cytb - 23 Hap and COI - 18 Hap), the COI marker better separated the species than Cytb. Cytochrome b was not able to separate the species *P. orbignyi* and *P. scobina* from *P. leopoldi*, despite that COI did not demonstrate expressive barcode gap. Generally in Barcode region, 2% is considered as the minimum interspecific limit (Hebert et al. 2003, Ward et al. 2005). However, in the case of freshwater stingrays, in some comparisons between species with well-defined morphology, interspecific values less than 2% were found. This can be related to the natural history of the group, where the species of *Potamotrygon* genus diverged very recently (Lovejoy 1997, Marques 2001, not published in indexed journals, Toffoli et al. 2008).

Beyond the hybridization possibility and strong evidence of speciation in

Potamotrygonidae from Xingu river, the most remarkable result observed in our study was the divergence among haplotypes of *P. motoro* from Xingu and this *P. motoro* from Paraná-Paraguay river basin (sequences available in GenBank). Values above 2% were noticed, validating the last taxonomic revision of this species, which may have different lineages distributed over different river basins (Loboda & Carvalho 2013). It is possible to have more events of speciation occurring in Xingu river in *Potamotrygon* genus, as well as in *Paratrygon aiereba*.

Speciation in *Paratrygon*

Being that the intraspecific divergence inside *Paratrygon* was bigger than the interspecific mean distance in *Potamotrygon* suggests that *Paratrygon* have more than one species. This high divergence inside this monotype genus is congruent with other molecular data from ATPase subunit 6 and COI genes from 63 specimens (Frederico et al. 2012). Frederico et al. (2012) separated this species into three groups: (1) Solimões-Amazonas-Estuary System (SAE) and the Negro River (NEG); (2) the Xingu river group (XIN); (3) and another group from Araguaia river (ARA). Our data demonstrated subdivision into the group 2, proposing two new species which occurs in the Xingu river basin.

CONCLUSION

Fragments of 5' portion of mitochondrial genes COI and Cytb were used as Barcode sequences to identify the species of Potamotrygonidae from Xingu river basin. We found strong haplotypes sharing between species. Having different species according to morphological traits in the same group, in other words, there was the absence of reciprocal monophyly to the majority of the taxa. This was the first record of hybridization in freshwater stingrays from Xingu river basin.

Along with this important hybridization evidence, we found profound divergence among haplotypes from the same species, which indicates speciation in the species. As in the case of *P. motoro* (from this study) and sequences from Paraná river (Pereira et al. 2013). Furthermore, the high values of genetic distance between the haplotypes of *Paratrygon aiereba* suggest speciation in this genus, indicating the possibility to include a new species to Xingu.

Hybridization clues allied with the unsolved taxonomy of freshwater stingrays, as well as its recent diversification, may collaborate to the lack of efficiency of the mitochondrial markers as Barcode tool (Toffoli et al. 2008), as confirmed in the present study. Nonetheless, the approaches performed were very successful in the separation between genera, as observed over the Barcode gap in the comparisons taken. Additionally, it could show the hidden cryptic diversity on Potamotrygonidae, revealing also strong evidence of speciation in *Paratrygon* genus. Therefore, this shows that either the COI gene, the official Barcode region for fishes and other animals (Hebert et al. 2003, Ward et al. 2005), as Cytb can be successfully used for accurate species identification, once the family taxonomy under investigation is well understood.

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