RESEARCH ARTICLE

Hidden diversity in *Prochilodus nigricans*: A new genetic lineage within the Tapajós River basin

Ueslei Lopes1,2*, Pedro M. Galetti, Jr.1, Patricia Domingues de Freitas1

1 Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos, SP, Brazil,
2 Centro de Ciências da Natureza, Campus Lagoa do Sino, Universidade Federal de São Carlos, Buri, SP, Brazil

* uesleilopes@gmail.com

Abstract

Highly spread through the Amazon River basin, *Prochilodus nigricans* have had its taxonomic validity recently questioned, when genetic differences between Western and Eastern Amazon populations from the Brazilian shield were detected. This area has been seeing as a region of high ichthyofaunal diversity and endemism, in which the hybrid origin of the Tapajós River basin has been raised. In this paper, we report a new molecular lineage within *P. nigricans* of Tapajós River, highlighting this region still hides taxonomically significant diversity. Haplotype networks were reconstructed using the mitochondrial COI and ATP6/8 markers, which were also used to calculate genetic distances among clusters. We additionally conducted a delimiting species approach by employing a Generalized Mixed Yule-Coalescent model (GMYC) with COI sequences produced here, and previous ones published for individuals sampled across the Amazon River basin. In addition to the genetic differentiation within *P. nigricans*, our findings favor the hypothesis of hybrid origin of the Tapajós River basin and reaffirm the importance of studies aiming to investigate hidden diversity to address taxonomic and biogeographic issues, that certainly benefit better biodiversity conservation actions.

1. Introduction

Freshwater ecosystems are exposed to great human-promoted impacts and transformations [1], making studies focusing on the discovery and comprehension of the extent biodiversity crucial for their conservation [2,3]. With over 5,160 freshwater fish species described in the South American rivers, this region harbors one-third of fish species of the entire planet, and the expectation is that this number is 42% higher [4]. Under this perspective, the Amazon River basin occupies a remarkable position, since its large extension is home for a huge diversity of fish species [5], many of which remain unknown.

Among the fish diversity from the Amazon basin, *Prochilodus nigricans* (Prochilodontidae, Characiformes) is one of the three species of the genus found in the Amazon River basin and presents the largest geographic distribution through the drainage in comparison with its
congeners [6]. Known as Black prochilodus or curimbata, *P. nigricans* is an abundant species that initiates spawning migration as soon as the flooding season starts [7,8]. With a detritivorous diet, this fish plays an essential functional role in ecosystems by modulating the fluxes of energy and nutrients [9–12]. *P. nigricans* also assumes an important economic and social role in Brazil, since it is one of the dominant species in local fisheries and highly used by the riverine community for subsistence [13,14].

Recently, studies on molecular phylogeny have questioned the monophyly of *P. nigricans* pointing out the existence of two mitochondrial lineages in the Amazon River basin [15,16]. A lineage includes specimens of *P. nigricans* from lowlands of Western Amazon and its mainstream, while a second one is considered a complex of species that includes what is described as *P. nigricans* from uplands of the Eastern Amazon (Araguaia River, Upper and Middle Tapajós River), *P. britskii* from Apiacás (Upper Tapajós River), *P. brevis* from northeastern Brazil (Ceará and Rio Grande do Norte states), *P. lacustris* from Parnaíba River, and *P. rubrotaeniatas* from the Upper Orinoco and Upper Essequibo River basins [15,16]. Namely, within this complex lineage two taxonomic units, *P. britskii* and the *P. nigricans* Eastern Amazon group, were also found in the Tapajós River basin.

The Amazon biogeography is quite complex, and this is particularly evident in the Tapajós River basin. Distinct cladistic approaches and a broad sampling across the Amazon showed an intricate history for this hydrographic system, in which the Tapajós River basin was depicted as non-monophyletic, showing a high degree of historical hybridism [17]. In this scenario, the occurrence of new taxonomic units into the Tapajós River basin can be expected. Considering the Brazilian Shield is an underestimated region of high ichthyofaunal diversity and endemism [18], and the previously reported *Prochilodus* phylogeny [15,16] included few individuals from Tapajós River, this basin still requires a more extensive sampling.

In this sense, and taking into account the importance of DNA-based approaches for delimiting species [5,19–22], we analyzed *P. nigricans* throughout the Tapajós River basin to investigate if this hydrographic system still hides taxonomically significant diversity for this important fishery resource, which represents the third most captured taxon (in tons) in the Brazilian Amazon River basin [23]. We implemented COI and ATP6/8 molecular analyses and used well-established algorithms for species-delimitation analyses. Our data raised a new Molecular Operational Taxonomic Units (MOTUs) within *P. nigricans* and certainly contributes for better estimating of biodiversity into the taxon.

**2. Material and methods**

**2.1. Study area**

The Tapajós River basin (Fig 1) is one of the largest watersheds constituting the Amazon River basin, encompassing an area of 493,986 hectares [24] and discharging approximately 6.4% of all water carried to the Amazon River [25]. This drainage hosts portion of Amazonian and Cerrado biomes, being also recognized as a peculiar ecoregion [26]. Located at the Brazilian Shield western portion, the Tapajós is a 795 km long clearwater river formed by the confluence of the Juruena and Teles Pires tributaries, whose present the length of 1240 km and 1457 km, respectively [27–29].

**2.2. Biological sampling and ethical requirements**

This study was carried out in accordance with the Brazilian law for environmental protection under the license for fish collection (SISBIO 41778–7), access of genetic material (SISGEN AAA03B9), and was approved by the Animal Ethics Committee of the Universidade Federal de São Carlos (CEUA/UFSCar 3752060715).
Biological samples of *P. nigricans* from the Tapajós River basin were collected during 2015. We sampled small fragments of fin tissue from adult specimens only, and most of the fish were returned alive to the river. Fin samples were additionally provided by local fishermen. We also obtained fin tissue samples from other Amazon rivers through collaborators and scientific collections (Table 1). All tissue samples were preserved in alcohol 95%, and species identification was performed based on morphological criteria, following Castro and Vari [6]. When available, new vouchers were deposited into the biological collection of the Laboratório de Ictiologia e Sistemática at Universidade Federal de São Carlos (LISDEBE/UFSCar, São Carlos, SP). Further information on this dataset, including available vouchers and Genbank (https://www.ncbi.nlm.nih.gov/nucleotide/) accession numbers are provided in Table 1.

In total, we analyzed 48 samples of *P. nigricans*. From this total, 38 were collected through the Tapajós River basin: 28 from the Tapajós River main channel, and nine from first and second-order tributaries (six from Juruena, one from Teles Pires, and two from Apiacas rivers). Ten samples were obtained from the Xingu (1) and Tocantins-Araguaia (9) drainages (Fig 1, Table 1). Additionally, we retrieved 48 sequences of *P. nigricans* and some congeneric nominal species from previous studies [15,16,30–32], available at Genbank and Barcode of Life Data Base (BOLD Systems, https://www.boldsystems.org/index.php/databases) public databases (S1 Table).
Table 1. Sampling information.

| ID | River       | Locality, State      | Accession Number | COI       | ATP6/8     |
|----|-------------|----------------------|------------------|-----------|------------|
| A1 | Arinos¹      | Juara, MT            | MN996677         | MT052031  |
| A2 | Arinos¹      | Juara, MT            | MN996681         | MT052032  |
| A3 | Arinos¹      | Juara, MT            | MN996682         | MT052033  |
| B1 | Sangué¹     | Juara, MT            | MN996679         | MT052035  |
| B2 | Sangué¹     | Juara, MT            | MN996678         | MT052036  |
| D1 | Tapajós     | Itaituba, PA         |                 | MT052043  |
| D2 | Tapajós     | Itaituba, PA         | MN996695         | MT052051  |
| D3 | Tapajós     | Itaituba, PA         | MN996685         | MT052041  |
| D4 | Tapajós     | Itaituba, PA         | MN996686         | MT052042  |
| D5 | Tapajós     | Itaituba, PA         | MN996692         | MT052040  |
| D6 | Tapajós     | Itaituba, PA         | MN996698         | MT052053  |
| D7 | Tapajós     | Itaituba, PA         |                 | MT052054  |
| D8 | Tapajós     | Itaituba, PA         |                 | MT052045  |
| D9 | Tapajós     | Itaituba, PA         | MN996701         | MT052059  |
| D10| Tapajós     | Itaituba, PA         |                 | MT052049  |
| D11| Tapajós     | Itaituba, PA         | MN996696         | MT052057  |
| D12| Tapajós     | Itaituba, PA         |                 | MT052055  |
| D13| Tapajós     | Itaituba, PA         |                 | MT052050  |
| D14| Tapajós     | Itaituba, PA         |                 | MT052044  |
| D15| Tapajós     | Itaituba, PA         |                 | MT052052  |
| D16| Tapajós     | Itaituba, PA         | MN996684         | -         |
| D17| Tapajós     | Itaituba, PA         | MN996700         | -         |
| D18| Tapajós     | Itaituba, PA         | MN996697         | -         |
| D19| Tapajós     | Itaituba, PA         | MN996690         | -         |
| D20| Tapajós     | Itaituba, PA         | MN996688         | -         |
| D21| Tapajós     | Itaituba, PA         | MN996689         | -         |
| D22| Tapajós     | Itaituba, PA         | MN996691         | -         |
| D23| Tapajós     | Itaituba, PA         | MN996694         | -         |
| E1 | Teles Pires  | Sorriso, MT          | MN996693         | MT052037  |
| I1 | Apiacás²    | Alta Floresta, MT    |                 | MT052039  |
| J1 | Apiacás²    | Alta Floresta, MT    | MN996687         | -         |
| F1 | Teles Pires  | Cotriguaçu, MT       | MN996683         | MT052038  |
| XI01| Curuá³     | Altamira, PA         | MN996674         | -         |
| TA04| Araguaia   | Barra do Garça, MT   | MN996675         | -         |
| TA06| Araguaia   | Barra do Garça, MT   | MN996676         | -         |
| TA07| Araguaia   | Barra do Garça, MT   | MN996673         | -         |
| TA15| Araguaia   | Barra do Garça, MT   | MN996671         | -         |
| TA17| Araguaia   | Barra do Garça, MT   | MN996672         | -         |
| TA18| Araguaia   | Barra do Garça, MT   | MN996668         | -         |
| TA43| Tocantins  | Palmas, TO           | MN996670         | -         |

(Continued)
2.3. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted following the saline precipitation protocol described by Aljanabi & Martinez [33]. Each DNA sample was quantified using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany), and standardized aliquots at 50 ng/μL were prepared. Polymerase Chain Reaction (PCR) was carried out in order to amplify the COI and ATP synthase subunit six (ATPase6) and eight (ATPase8) genes of the mitochondrial DNA (mtDNA). The primers Fish F1 and Fish R1, and ATP 8.2_L8331 and CO3.2_H9236 were used to amplify COI and ATP6/8 regions, respectively [34,35].

Polymerase chain reactions consisted of 50 ng of DNA template, 1.25 μL of buffer (10x), 0.2 mM of dNTPs, 1 mM of MgCl2, 0.4 μM of each primer, 0.5 U Taq Platinum (Invitrogen™) and ultra-pure distilled water to make up a final volume of 12.5 μL. DNA amplification reactions were carried out in an Applied Biosystems Veriti® 96-Well Thermal Cycler under the following conditions: COI—1 cycle [94˚C/2 min], 35 cycles [94˚C/30 sec, 59˚C/30 sec, 72˚C/1 min], 1 cycle [72˚C/10 min], and ATP6/8, 1 cycle [94˚C/3 min], 30 cycles [94˚C/45 sec, 58˚C/1 min, 72˚C/1 min], 1 cycle [72˚C/2 min]. PCR products were purified with 20% Polyethylene glycol (PEG) protocol [36] to remove unincorporated dNTPs and the excess of primers or unspecific bands. Sequencing was performed in an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, California, USA).

2.4. Data analyses

After sequencing, we first aligned and edited the data using ClustalW [37] and Geneious v.7.1.7 (Biomatters, Auckland, New Zealand) [38], respectively. Median Joining haplotype networks for both COI and ATP6/8 markers were reconstructed using PopART (Population Analysis with Reticulate Trees) [39]. We included 22 public sequences of *P. nigricans* and congeneric nominal species from the Brazilian Shield for the COI analyses. MEGA v.7 [40] was used to perform maximum-likelihood (ML) under HKY+I model and Neighbor-Joining (NJ) based on Kimura-2-parameters (K2P) analyses, both with 1000 of bootstrap replicates. Intra and intergroups genetic distances, based on K2P, with samples collected in both the Tapajós River basin and Eastern Amazon area were estimated also using the MEGA v.7 software.

General Mixed Yule Coalescent (GMYC) [41] approach was implemented for COI sequences in R with the SPLITS package (SPecies’ LImits by Threshold Statistics) [42], considering the single threshold under the default settings (interval = c(1,10)). GMYC combines stochastic lineage growth models with coalescence ones to detect intra and interspecific evolutionary processes (coalescence and speciation/extinction events, respectively) and has...
been commonly applied to identify MOTUs in studies concerning taxonomic issues [43–46].
For this analysis, a COI ultrametric topology was produced using the Bayesian Inference (BI) method implemented by BEAST v.2.0 [47] in CIPRES Science Gateway [48] (www.phylo.org). We also used the site model, based on Bayesian Information Criteria (BIC), as suggested by JModeltest [49] (COI = HKY+I), and lognormal relaxed molecular clock model and birth-death tree prior, according to Costa-Silva et al. [50]. Two independent runs of four Markov chains of 120 million generations were conducted, sampling every 10000 steps, with 30% of the first topologies being discarded as burn-in. The combination of the independent tree and log files was performed with the LogCombiner v.1.8 software [51] and stationarity and convergence were assessed with Tracer v1.5 [52], considering values of the effective sample size (ESS) of all parameters equal or higher than 200. A maximum clade credibility tree was summarized in TreeAnnotator v.1.8 [53], and later visualized in the FigTree v.1.4 software, which is available at http://tree.bio.ed.ac.uk/software/figtree/.

GMYC, ML, and NJ analyses were performed gathering all COI dataset, which comprised the newly generated sequences in addition to those downloaded from the databases.

3. Results and discussion

The total dataset generated in this study consisted of 35 COI and 29 ATP6/8 sequences, after alignment and editing (Table 1). COI fragments ranged from 561 to 564 bp and presented 26 polymorphic sites and 16 parsimony informative ones. The average ATP6/8 sequence length was 985 bp and included 18 variable sites, 11 of which were parsimony informative. The COI and ATP6/8 networks included 19 and 12 haplotypes, respectively, revealing individuals within the Tapajós mainstream assigned to highly divergent haplotypes, showing at least nine and six mutational steps from the most frequent haplotype for COI and ATP6/8, respectively (See S1 Fig).

The maximum likelihood results of the GMYC model performed with COI was significantly higher (L = 696.0997) than that of the null model (L0 = 642.3439), allowing us to reject the hypothesis that all individuals belong to the same molecular unit. The GMYC single threshold analysis revealed the occurrence of three MOTUs within the Amazon River basin (Fig 2), increasing the number of MOTUs reported in previous studies [15,16] in this large basin. Our study recovered the previous Western and Eastern Amazon MOTUs (showed, respectively, in blue and green clusters in Fig 2) and raised an additional one, named hereafter Tapajós MOTU (orange cluster in Fig 2).

The pairwise K2P distances between this new molecular lineage and rest of the Eastern group were 0.017 ± 0.004 and 0.01 ± 0.003 for COI and ATP6/8, respectively. Although both values were lower than that commonly used as an initial threshold for the molecular identification approach [54], our distance values were similar to those found between other Prochilodus species, which ranged from 1.2% to 10.3% [16]. The distances within groups using the COI data were 0.011 ± 0.002 for the Eastern group and 0.002 ± 0.001 for the new MOTU, while with ATP6/8 it ranged from 0.001 ± 0.000 and 0.002 ± 0.001 for the Eastern group and the new MOTU, respectively.

It is noteworthy that two of the P. nigricans MOTUs observed here are co-occurrent into the Tapajós River, and in the phylogenetic tree were shown as paraphyletic (Fig 2). This result corroborates previous biogeographic findings, in which the Tapajós River basin was seen as non-monophyletic, indicating a possible hybrid origin of this hydrographic system [17]. As stated by Dagosta and de Pinna [17], the Tapajós mainstream is related to the rivers of the Western Amazon, while the Juruena and Teles Pires tributaries are related to the Eastern drainages of the Brazilian shield. The same pattern of relationship was here verified among the
P. nigricans lineages through the GMYC analysis (Fig 2), which was also well supported by the ML (>79) and NJ (>74) topologies (S2 Fig). The Tapajós and Western Amazon lineages come up as sister groups, whereas the samples of P. nigricans collected within the tributaries of the Tapajós River (See Table 1 and S1 Table) represented the Eastern Amazon lineage. It is worthy to note that individuals of both Tapajós and Eastern Amazon lineages were seen in sympatry at least in part of the collection sites. Given the entanglement of the Amazon River basin, diverse phylogeographic hypotheses [55,56] have been hitherto postulated to explain it. Within this framework, and considering our results, P. nigricans raises a possible and interesting model to test such ideas. Different migratory fish groups such as Brycon [57], Leporinus [5], and Zungaro [19] have been showing hidden diversity, particularly in the Tapajós River basin. Overall, these results appear indicating both the high level of endemism [18] and the putative historical hybridism [6] can be important drives of fish diversity in this peculiar system.

Our findings reinforce the importance of molecular species delimitation approach in investigating hidden biodiversity into the Amazon ichthyofauna. Since the Amazon River basin is in the spotlight as a candidate area for the construction of diverse hydroelectric plants [58,59] and threatened by other factors, such as overharvesting, deforestation and climate change [13,60,61], the development of studies focusing on biodiversity survey is of paramount importance for effective conservation management plans [3,62,63], aiming at maintaining the maximum genetic diversity and evolutionary potential for a species [64,65].

Overall, the present study was able to reveal hidden biodiversity in P. nigricans, delimitating genetic lineages, and helping to characterize a new MOTU that must be better investigated to confirm the existence of a new species. Moreover, our findings raise new insights for a further approach related to the possible historical hybrid origin of the Tapajós River basin.

Supporting information

S1 Fig. Haplotype networks using samples from the Tapajós River basin and the Eastern Amazon clade. A. COI, B. ATP6/8.
(TIF)

S2 Fig. COI topologies including the generated data and the sequences retrieved from public databases. A. Maximum likelihood, B. Neighbor joining.
(TIF)

S1 Table.
(DOCX)

Acknowledgments

The authors thank Solange Arrolho, Miliany Campos, Paulo Cesar Venere, Marcelo Brito, Ademir and Erzidio for collecting/donating samples. Michel Gianeti and Mario de Pinna (MZUSP) are acknowledged for loaning tissues. We also thank Pedro Gallo and Bruno Saranholi for the field assistance. Carla Gestich, Josiane Ribolli and Jorge Ramirez are thanked for the contributions. Editor and referees are also acknowledged for contributing to the improvement of this manuscript.
Author Contributions

Conceptualization: Ueslei Lopes, Pedro M. Galetti, Jr., Patricia Domingues de Freitas.

Data curation: Patricia Domingues de Freitas.

Formal analysis: Ueslei Lopes, Pedro M. Galetti, Jr..

Funding acquisition: Pedro M. Galetti, Jr., Patricia Domingues de Freitas.

Writing – original draft: Ueslei Lopes.

Writing – review & editing: Ueslei Lopes, Pedro M. Galetti, Jr., Patricia Domingues de Freitas.

References

1. Dudgeon D, Arthington AH, Gessner MO, Kawabata Z, Naiman RJ, Knowler DJ, et al. Freshwater biodiversity: importance, threats, status and conservation challenges. Biol Rev. 2006; 81: 163–182. https://doi.org/10.1017/S1464793105006950 PMID: 16336747

2. Alofs KM, Liverpool EA, Taphorn DC, Bernard CR, López-Fernández H. Mind the (information) gap: the importance of exploration and discovery for assessing conservation priorities for freshwater fish. Divers Distrib. 2014; 20: 107–113. https://doi.org/10.1111/ddi.12127

3. Sales NG, Mariani S, Salvador GN, Pessali TC, Carvalho DC. Hidden Diversity Hampers Conservation Efforts in a Highly Impacted Neotropical River System. Front Genet. 2018; 9: 1–11. https://doi.org/10.3389/fgene.2018.00001

4. Reis RE, Albert JS, Di Dario F, Mincarone MM, Petry P, Rocha LA. Fish biodiversity and conservation in South America. J Fish Biol. 2016; 89: 12–47. https://doi.org/10.1111/jfb.13016 PMID: 27312713

5. Silva-Santos R, Ramirez JL, Galetti PM, Freitas PO. Molecular Evidences of a Hidden Complex Scenario in Leporinus cf. friderici. Front Genet. 2018; 9: 47. https://doi.org/10.3389/fgene.2018.00047 PMID: 29497440

6. Castro RMC, Vari RP. Detritivores of the South American fish family Prochilodontidae (Teleostei:Ostariophysi:Characiformes): a phylogenetic and revisionary study. Smithsonian Contrib to Zool. 2004; 1–189. https://doi.org/10.5479/si.00810282.622

7. Loubens G, Panfilii J. Biologie de Prochilodus nigricans (Teleostei: Prochilodontidae) dans le bassin du Mamoré (Amazonie bolivienne). Ichthyol Explor Freshwaters. 1995; 6: 17–32. Available from: http://www.documentation.ird.fr/hor/doi:42211

8. Montreuil V, García Á, Rodríguez R. Biología reproductiva de “Boquichico” Prochilodus nigricans, en la amazonia peruana. Folia Amaz. 2001; 12: 5–14.

9. Stassen MJM, van de Ven MWPM, van der Heide T, Hiza MAG, van der Velde G, Smolders AJP. Population dynamics of the migratory fish Prochilodus lineatus in a neotropical river: the relationships with river discharge, flood pulse, El Niño and fluvial megafan behaviour. Neotrop Ichthyol. 2010; 8: 113–122. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1679-62252010000100014&nrm=iso

10. Flecker AS. Ecosystem Engineering by a Dominant Detritivore in a Diverse Tropical Stream. Ecology. 1996; 77: 1845–1854.

11. Taylor BW, Flecker AS, Hall RO. Loss of a harvested fish species disrupts carbon flow in a diverse tropical river. Science (80-). 2006; 313: 833–836. https://doi.org/10.1126/science.1128223 PMID: 16902137

12. Bonilla-Castillo CA, Córdoba EA, Gómez G, Duponchelle F. Population dynamics of Prochilodus nigricans (Characiformes: Prochilodontidae) in the Putumayo River. Neotrop Ichthyol. 2018; 16: 1–12. https://doi.org/10.1590/1982-0224-20170139

13. Castelló L, McGrath DG, Hess LL, Cole MT, Lefebvre PA, Petry P, et al. The vulnerability of Amazon freshwater ecosystems. Conserv Lett. 2013; 6: 217–229. https://doi.org/10.1111/conl.12008

14. Fernandes CC. Lateral migration of fishes in Amazon floodplains. Ecol Freshw Fish. 1997; 6: 36–44. https://doi.org/10.1111/j.1600-0633.1997.tb00140.x

15. Melo BF, Sidauskas BL, Hoekzema K, Frable BW, Vari RP, Oliveira C. Molecular phylogenetics of the Neotropical fish family Prochilodontidae (Teleostei: Characiformes). Mol Phylogenet Evol. 2016; 102: 189–201. https://doi.org/10.1016/j.ympev.2016.05.037 PMID: 27262428

16. Melo BF, Dorini BF, Foresti F, Oliveira C. Little Divergence Among Mitochondrial Lineages of Prochilodus (Teleostei, Characiformes). 2018; 9: 1–9. https://doi.org/10.3389/fgene.2018.00107 PMID: 29670644
17. Dagosta FCP, de Pinna MCC. Biogeography of Amazonian fishes: deconstructing river basins as biogeographical units. Neotrop Ichthyol. 2017; 15: 1–24. https://doi.org/10.1590/1982-0224-20170034

18. Machado VN, Collins RA, Ota RP, Andrade MC, Farias IP, Hrbek T. One thousand DNA barcodes of piranhas and pacus reveal geographic structure and unrecognised diversity in the Amazon. Sci Rep. 2018; 1–12. https://doi.org/10.1038/s41598-017-17765-5

19. Pires AA, Ramirez JL, Galetti PM, Troy WP, Freitas PD. Molecular analysis reveals hidden diversity in Zungaro (Siluriformes: Pimelodidae): a genus of giant South American catfish. Genetica. 2017; 145: 335–340. https://doi.org/10.1007/s10709-017-9968-8 PMID: 28501957

20. Serrano ÉA, Melo BF, Freitas-Souza D, Oliveira MLM, Utsunomia R, Oliveira C, et al. Species delimitation in Neotropical fishes of the genus Characidium (Teleostei, Characiformes). Zool Scr. 2019; 48: 69–80. https://doi.org/10.1111/zsc.12318

21. Santana CD De Crampton WGR, Dillman CB Frederico RG, Sabaj MH, Covain R, et al. Unexpected species diversity in electric eels with a description of the strongest living bioelectricity generator. Nat Commun. 2019; 1–10. https://doi.org/10.1038/s41467-018-07882-8

22. Garcia-Melo J, Oliveira C, Da Costa Silva G, Ochoa-Orrego L, Garcia Pereira L, Maldonado-Ocampo J. Species delimitation of neotropical Characins (Stevatiinae): Implications for taxonomy of complex groups. PLoS One. 2019; 14: 1–22. https://doi.org/10.1371/journal.pone.0216786

23. Araujo-Lima CARM, Ruffino ML. Migratory Fishes of the Brazilian Amazon. In: Carolsfeld J, Harvey B, Lis JT, Schleif R. Size fractionation of double-stranded DNA by precipitation with polyethylene glycol. Nucleic Acids Res. 1975; 2: 383–390. https://doi.org/10.1093/nar/2.3.383 PMID: 236548

24. Latrubesse EM, Stevaux JC, Sinha R. Tropical rivers. Geomorphology. 2005; 70: 187–206. https://doi.org/10.1016/j.geomorph.2005.02.005

25. Abell R, Thieme ML, Revenga C, Bryer M, Kottelat M, Bogutskaya N, et al. Freshwater Ecoregions of the World: A New Map of Biogeographic Units for Freshwater Biodiversity Conservation. Bioscience. 2008; 58: 403. https://doi.org/10.1641/B580507

26. Ohara WM, Loeb MV. Ichthyofauna of the upper Juruena river on Chapada dos Parecis, Mato Grosso, Brazil. Biota Neotrop. 2016; 16: 1–10.

27. Instituto de Chico Mendes de Conservação da Biodiversidade. Plano de Manejo do Parque Nacional do Juruena. Encarte 2. 2011[cited 16 Jan 2020]. Available from: http://www.icmbio.gov.br/portal/images/stories/imgs-unidades-conservacao/Encarte2.pdf

28. Souza DF de, Bermann C, Fonseca CR, Silva EAS da. UHE Teles Pires: um estudo de caso de geração hidrelétrica na Amazônia. Rev Geoaraguaia. 2016; 6: 95–111. Available from: http://periodicoscientificos.ufmt.br/ojs/index.php/geo/article/view/4907/pdf_1

29. Ardura A, Linde AR, Moreira JC, Garcia-Vazquez E. DNA barcoding for conservation and management of Amazonian commercial fish. Biol Conserv. 2010; 143: 1438–1443. https://doi.org/10.1016/j.biocon.2010.03.019

30. Ardura A, Planes S, Garcia-Vazquez E. Applications of DNA barcoding to fish landings: Authentication and diversity assessment. Zookeys. 2013; 365: 49–65. https://doi.org/10.3897/zookeys.365.6409 PMID: 24453550

31. Ardura A, Gomes V, Linde AR, Moreira JC, Hrbek T, Garcia-Vazquez E. The Meeting of Waters, a possible shelter of evolutionary significant units for Amazonian fish. Conserv Genet. 2013; 14: 1185–1192. https://doi.org/10.1007/s10709-013-9968-8 PMID: 28501957

32. Aljanabi SM, Martinez I. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Res. 1997; 25: 4692–4693. https://doi.org/10.1093/nar/25.22.4692 PMID: 9358185

33. Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN. DNA barcoding Australia’s fish species. Philos Trans R Soc Lond B Biol Sci. 2005; 360: 1847–1857. https://doi.org/10.1098/rstb.2005.1716 PMID: 16214743

34. Sivasundar A, Bermingham E, Orti G. Population structure and biogeography of migratory freshwater fishes (Prochilodus: Characiformes) in major South American rivers. Mol Ecol. 2001; 10: 407–417. https://doi.org/10.1046/j.1365-294x.2001.01194.x PMID: 11298955

35. Lis JT, Schleif R. Size fractionation of double-stranded DNA by precipitation with polyethylene glycol. Nucleic Acids Res. 1975; 2: 383–390. https://doi.org/10.1093/nar/2.3.383 PMID: 236548

36. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007; 23: 2947–2948. https://doi.org/10.1093/bioinformatics/btm404 PMID: 17846036
38. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012; 28: 1647–1649. https://doi.org/10.1093/bioinformatics/bts199 PMID: 22543367

39. Leigh JW, Bryant D. POPART: Full-feature software for haplotype network construction. Methods Ecol Evol. 2015; 6: 1110–1116. https://doi.org/10.1111/2041-210X.12410

40. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016; 33: 1870–1874. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

41. Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S, et al. Sequence-Based Species Delimitation for the DNA Taxonomy of Undescribed Insects. Syst Biol. 2006; 55: 595–609. https://doi.org/10.1080/10635150600852011 PMID: 16967577

42. Monaghan MT, Wild R, Elliot M, Fujisawa T, Balke M, Inward DJG, et al. Accelerated species Inventory on Madagascar using coalescent-based models of species Delination. Syst Biol. 2009; 58: 298–311. https://doi.org/10.1093/sysbio/syp027 PMID: 20525585

43. Low VL, Takaoka H, Pramual P, Adler PH, Ya’cob Z, Huang Y-T, et al. Delineating taxonomic boundaries in the largest species complex of black flies (Simulidae) in the Oriental Region. Sci Rep. 2016; 6: 20346. https://doi.org/10.1038/srep20346 PMID: 26839292

44. Machado CDB, Ishizuka TK, Freitas PD De, Valiati VH, Galetti PM. DNA barcoding reveals taxonomic uncertainty in Salminus (Characiformes). Syst Biodivers. 2017; 15: 372–382. https://doi.org/10.1080/14772000.2016.1254390

45. Mills S, Alcántara-Rodríguez JA, Ciros-Pérez J, Gómez A, Hagiwara A, Galindo KH, et al. Fifteen species in one: deciphering the Brachionus plicatilis species complex (Rotifera, Monogononta) through DNA taxonomy. Hydrobiologia. 2017; 796: 39–58. https://doi.org/10.1007/s10750-016-2725-7

46. Vacher JP, Kok PJR, Rodrigues MT, Lima JD, Lorenzini A, Martinez Q, et al. Cryptic diversity in Amazonian frogs: Integrative taxonomy of the genus Anomaloglossus (Amphibia: Anura: Aromobatidae) reveals a unique case of diversification within the Guiana Shield. Mol Phylogenet Evol. 2017; 112: 158–173. https://doi.org/10.1016/j.ympev.2017.04.017 PMID: 28438699

47. Bouckaert R, Heled J, Kühnert D, Vaughan T, Xie D, et al. BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. PLoS Comput Biol. 2014; 10: 1–6. https://doi.org/10.1371/journal.pcbi.1003537 PMID: 24722319

48. Costa-Silva GJ, Rodriguez MS, Roxo FF, Foresti F, Oliveira C. Using different methods to access the difficult task of delimiting species in a complex neotropical hypervarid fauna. BMC Genet. 2013; 14: 20. https://doi.org/10.1186/1471-2156-14-20 PMID: 23497346

49. Hubert N, Renno JF. Historical biogeography of South American freshwater fishes. J Biogeogr. 2006; 33: 1414–1436. https://doi.org/10.1111/j.1365-2699.2006.01518.x

50. Leite RN, Rogers DS. Revisiting Amazonian phylogeography: Insights into diversification hypotheses and novel perspectives. Org Divers Evol. 2013; 13: 639–664. https://doi.org/10.1007/s13127-013-0140-8

51. Arruda PSS, Ferreira DC, Oliveira C, Venere PC. DNA barcoding reveals high levels of divergence among mitochondrial lineages of Brycon (Characiformes, bryconidae). Genes (Basel). 2019; 10: 5–7. https://doi.org/10.3390/genes10090639 PMID: 31450860

52. Tundisi JG, Goldemberg J, Matsumura-Tundisi T, Saraiva ACF. How many more dams in the Amazon? Energy Policy. 2014; 74: 703–708. https://doi.org/10.1016/J.ENPOL.2014.07.013

53. Latrubesse EM, Arima EY, Dunne T, Park E, Baker VR, d’Horta FM, et al. Damming the rivers of the Amazon basin. Nature. 2017; 546: 363–369. https://doi.org/10.1038/nature22333 PMID: 28617466
60. Guimberteau M, Ciais P, Pablo Boisier J, Paula Dutra Aguiar A, Biemans H, De Deurwaerder H, et al. Impacts of future deforestation and climate change on the hydrology of the Amazon Basin: A multi-model analysis with a new set of land-cover change scenarios. Hydrol Earth Syst Sci. 2017; 21: 1455–1475. https://doi.org/10.5194/hess-21-1455-2017

61. Val AL, Fearnside PM, Almeida-Val VMF. Environmental disturbances and fishes in the Amazon. J Fish Biol. 2016; 89: 192–193. https://doi.org/10.1111/jfb.12896 PMID: 26864975

62. Uriarte M, Erickson DL, Kress WJ, García C. DNA barcodes for ecology, evolution, and conservation. Trends Ecol Evol. 2014; 1–11. https://doi.org/10.1016/j.tree.2014.10.008 PMID: 25468359

63. Ely CV, Bordignon SA de L, Trevisan R, Iob I, Boldrini. Implications of poor taxonomy in conservation. J Nat Conserv. 2017; 36: 10–113. https://doi.org/10.1016/j.jnc.2017.01.003

64. Martinez AS, Willoughby JR, Christie MR. Genetic diversity in fishes is influenced by habitat type and life-history variation. Ecol Evol. 2018; 8: 12022–12031. https://doi.org/10.1002/ece3.4661 PMID: 30598796

65. Manel S, Guérin PE, Mouillot D, Blanchet S, Velez L, Albouy C, et al. Global determinants of freshwater and marine fish genetic diversity. Nat Commun. 2020; 11: 1–9. https://doi.org/10.1038/s41467-019-13993-7