Electronic Supplementary Material 2

Molecular Biology Reports

**FishDNAIDs: DNA barcoding as a tool in the development of a detection system to four species of Characiformes of commercial importance in the Brazilian Amazon**

Danniel Rocha Bevilaqua*[1]; Jacqueline da Silva Batista[2]; Adolfo José da Mota[3]; Ana Caroline Viana da Silva[4]; Andreza Mikeyne Silva da Mota[5]; Kyara Martins Formiga[6]; Carlos Edwar de Carvalho Freitas[7]

[1]Instituto Federal de Educação Ciência e Tecnologia do Amazonas (IFAM) – Campus Avançado Manacapuru, AM, Brazil.

[2]Coordenação de Biodiversidade (COBIO/INPA), Laboratório Temático de Biologia Molecular (LTBM/INPA), Programa de Pós-Graduação em Genética, Conservação e Biologia Evolutiva (PPG-GCBEv/NPA). Manaus, AM, Brazil.

[3]Faculdade de Ciências Agrárias, Departamento de Ciências Fundamentais e Desenvolvimento Agrícola, Universidade Federal do Amazonas (UFAM), Manaus, AM, Brazil.

[4]Programa de Pós-Graduação em Genética, Conservação e Biologia Evolutiva (GCBEVI/INPA), Manaus, AM, Brazil.

[5]Programa de Pós-Graduação em Biotecnologia e Recursos Naturais – PPG MBT, Universidade Estadual do Amazonas – UEA, Manaus, AM, Brazil.

[6]Coordenação de Biodiversidade (COBIO/INPA), Laboratório Temático de Biologia Molecular (LTBM/INPA), Programa de Pós-Graduação em Genética, Conservação e Biologia Evolutiva (PPG-GCBEv/NPA). Manaus, AM, Brazil.

[7]Faculdade de Ciências Agrárias, Departamento de Ciências Pesqueiras, Universidade Federal do Amazonas, [4]Programa de Pós-Graduação de Biologia de Água Doce e Pesca Interior (BADPI/INPA), Manaus, AM, Brazil. Orcid:

ORCID
Danniel Rocha Bevilaqua Orcid: 0000-0002-6043-7867
Jacqueline da Silva Batista Orcid: 0000-0002-6649-6200
Adolfo José da Mota Orcid: 0000-0002-9745-1128
Ana Caroline Viana da Silva Orcid: 0000-0002-8746-5008
Andreza Mikeyne Silva da Mota Orcid: 0009-0002-8243-5122

Kyara Martins Formiga Orcid: 0000-0002-3127-0447
Carlos Edwar de Carvalho Freita 0000-0001-5406-0998

* Corresponding author “Danniel Rocha Bevilaqua” danniel.bevilaqua@ifam.edu.br

**DNA extraction protocols, amplification, sequencing and data analysis for the construction of the DNA barcode sequences database**

The genomic DNA of fish specimens was extracted from muscle tissue using the commercial Wizard® Genomic DNA Purification (Promega Corporation, USA) extraction kit. Reagent conditions and final concentrations used in the approximately 650 bp PCR of the COI were as follows: 1X of the buffer, 0.2 mM dNTPs (Thermo Scientific), 2.0 mM $MgCl_2$, 0.33 μM of each primer (Ward et al., 2005), 0.067U Taq DNA polymerase (Invitrogen) and 6.0 ng of DNA and ultrapure water qsp 15 μL [1]. Thermocycling was performed as follows: 68 °C for 60 sec, (93 °C for 10 sec, 50 °C for 35 sec, 68 °C for 45 sec) for 35 cycles and a final hold at 68 °C for 7 minutes.

Amplicon were purified using the EXOSAP-IT (Thermo Scientific™). Bilateral nucleotide reaction sequencing was performed, using the BigDye Terminator v3.1 Cycle Kit® Sequencing (ThermoFisher), followed by the elimination of unincorporated reagents during thermocycling (EDTA/Ethanol precipitation). The product of such reaction was electroinjected into the Genetic Analyzer (ABI 3130xL ThermoFisher). All steps using kits since DNA extraction to sequencing followed the manufacturer’s recommendations for each kit.

The DNA sequences were compiled using Geneious 8.0.5 (https://www.geneious.com). The Specimen DNA barcode identification was carried out by similarity analyzes with sequences in publicly-available nucleotide (INSDC) databases using nucleotide BLAST [2] and BOLDSystems IDS Tools (http://v4.boldsystems.org/) [3] until March 10, 2023. DNA barcode sequences from 111 specimens belonging to 18 species were downloaded from GENBANK (Table S1) and added to the DNA database used to design the primers and probes. This method was adopted in order to increase intraspecific diversity for the design of the DNA minibarcode flanking primers.

**Primer validation protocol in Sanger sequencing**

No template control (NTC) was performed at least in triplicate for all the experiments. The reagent conditions used for the PCR were as follows: 1X PCR buffer, 0.3 mM dNTPs, 2.0 mM $MgCl_2$, 0.25 µM of each species-specific primer (PNIG-F and PNIG-R, PALT-F and PALT-R, TANG-F and TANG-R, and PRUT-F and PRUT-R), 0.067 U Taq DNA polymerase (Invitrogen) and 0.1 ng of DNA and ultrapure water qsp 10 µL. Thermocycling was performed as follows: 68 °C 60 sec, 35 cycles at 93 °C 10 sec, 60 °C 35 sec, 68 °C 60 sec and a final hold at 68 °C for 7 minutes.

The PCR result was checked on 1.8% agarose gel, stained with Diamond™ (Promega) and photo-documented with an imaging system (Alfa Mini System, ProteinSimple). The amplicon for each species samples (target and congenetic) was purified using EXOSAP-IT and the nucleotide sequencing was generated, with the forward primer of each designed set. DNA sequences were compared and aligned with the COI (minibarcodes) fragment obtained previously for each of the four species, and flanked by the primers designed to confirm it. This phase was peformed using Geneious 8.0.5 (https://www.geneious.com).

**Test the efficiency of the primers**

2X PowerUp™ SYBR™ Green Master Mix (ThermoFisher), 0.2 µM of each primer and 0.1 ng of DNA and ultrapure water qsp 10 µL. The qPCR conditions were: 50 °C for 2 min, 95 °C for 2 min followed by 35 cycles of 15 sec at 95 °C and 60 °C for 1 min in a QuantStudio6 Flex Real-Time thermocycler (ThermoFisher).

**Testing the efficiency of the probes**

The reagents and thermocycling conditions used were 1X of TaqMan® Gene Expression Master Mix (ThermoFisher), 0.25 µM of each species-specific primers and probe (5'FAM/MGB-NFQ3'), 0.1 ng of DNA and ultrapure water qsp 10 µL. The qPCR conditions were: 50 °C for 2 min, 95 °C for 2 min followed by 35 cycles of 15 sec at 95 °C and 60 °C for 1 min. The reaction was carried out in a thermal cycler (QuantStudio6 Flex Real-Time, ThermoFisher).

**REFERENCES**


1. Cunha-Machado AS, Farias IP, Hrbek T, et al (2022) Genetic differentiation and gene flow of the Amazonian catfish *Pseudoplatystoma punctifer* across the Madeira River rapids prior to the construction of hydroelectric dams. Hydrobiologia 849:29–46
2. Altschul SF, Gish W, Miller W, et al (1990) Basic local alignment search tool. J Mol Biol 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2
3. Ratnasingham S, Hebert PDN (2007) BARCODING: bold: The Barcode of Life Data System (http://www.barcodinglife.org). Mol Ecol Notes 7:355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x