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SHORT COMMUNICATION

Range expansion of *Hollandichthys multifasciatus* (Characiformes: Acestrorhamphidae) to the upper Iguaçu River basin, Paraná, Brazil

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ABSTRACT. *Hollandichthys multifasciatus* (Eigenmann & Norris, 1900) is a fish endemic to the Atlantic Forest, occurring primarily in well-preserved headwater streams from Rio de Janeiro to Santa Catarina, with isolated records in the upper rio Tietê. Here, we report the first record of the species in the upper Rio Iguaçu basin, Paraná, Brazil. Three specimens (39–87 mm SL) were collected from a tributary of the Rio Miringuava, municipality of São José dos Pinhais, state of Paraná, in June 2022. Identification was based on diagnostic morphological characters and mitochondrial COI gene analysis, compared with sequences available in the literature. Maximum Likelihood (ML) phylogenetic analyses and haplotype network construction revealed high similarity between the upper Iguaçu population and those from coastal drainages flowing into Paranaguá Bay, suggesting a history of headwater capture between neighboring basins. This phenomenon, associated with Quaternary tectonic reactivation in the Serra do Mar, is an important mechanism for the dispersal and diversification of the Neotropical ichthyofauna. This finding expands the known distribution of the genus and contributes to the understanding of the evolutionary history and connectivity among Atlantic Forest hydrographic basins.

KEYWORDS. Aquatic ecoregion, DNA barcode, freshwater fishes, river connectivity, Thayeriinae.

The Neotropical genus *Hollandichthys* Eigenmann, 1909 is endemic to small, preserved streams of the Atlantic Forest coastal plain, with a distribution extending from Rio de Janeiro to Rio Grande do Sul, including an isolated record in the upper rio Tietê, upper rio Paraná basin (Menezes et al. 2007; Marceniuk et al. 2011). At present, the genus comprises two valid species: *Hollandichthys taramandahy* Bertaco & Malabarba, 2013 and *Hollandichthys multifasciatus* (Eigenmann & Norris, 1900); however, molecular evidence indicates that *H. multifasciatus* may be more diverse than initially recognized (Thomaz et al. 2010).

In the Atlantic Forest, *H. multifasciatus* is easily recognized among other characiforms by a combination of morphological characters, including distinctive black stripes along the flanks, a short lateral line, and maxillary bones bearing teeth along their entire margin (Bertaco 2022). Unique primary sexual characters in males (Quágio-Grassiotto et al. 2012) and a body cavity between the pelvic and anal fins housing the anus and urogenital openings (Bertaco & Malabarba 2013) support a close relationship between *Hollandichthys* and *Rachoviscus*.

Hollandichthys multifasciatus commonly inhabits small, preserved Atlantic Forest streams, preferably in marginal

blackwater and whitewater areas with shallow waters and weak currents, feeding mainly on insects and allochthonous plant material (Abilhoa et al. 2009). Among the two species of the genus, *H. multifasciatus* has the widest distribution, ranging from the states of Santa Catarina to Rio de Janeiro (Bertaco 2022). Recent sampling efforts in the headwaters of the Rio Miringuava, upper Rio Iguaçu basin, revealed the presence of *H. multifasciatus*, representing the first record of the genus in this basin. In this study, we document this unprecedented occurrence and discuss possible explanations for the presence of the species outside its typical distribution in coastal basins.

Three specimens of *H. multifasciatus* (39–87 mm SL) were collected from a small tributary of the rio Miringuava, in the upper rio Iguaçu basin (25°38'13.75"S; 49°3'47.81"W), municipality of São José dos Pinhais, state of Paraná, in June 2022 (Fig. 1). Voucher specimens were deposited in the ichthyological collection of the Museu de História Natural Capão da Imbuia (MHNCI) under catalog numbers MHNCI 13125. Sampling was authorized by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA/SISBIO 10320).

To confirm the identification of individuals from the upper Rio Iguaçu and to better understand the phylogenetic relationships of this population, the mitochondrial COI gene was sequenced from two individuals from the upper Iguaçu and one from a coastal stream draining into Paranaguá Bay. Obtained sequences (GenBank accession numbers: PX441979, PX441980, PX441981) were compared with data from Thomaz et al. (2010). For genomic DNA extraction, 25 mg of muscle tissue were collected from each specimen using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's instructions. DNA quantification and quality assessment were carried out by spectrophotometry using a Tecan Infinite M200 Pro device.

Amplification of the COI gene was performed by Polymerase Chain Reaction (PCR) in a final volume of 20 µL, containing 4 µL of genomic DNA, 1× buffer from Platinum™ SuperFi II DNA Polymerase (Thermo Fisher), 0.2 mM dNTPs (Promega), 0.5 µM of the forward primer fishCO1F (5'-TCAACYAATCAYAAAGATATYGGCAC-3') and 0.5 µM of the reverse primer fishCO1R (5'-ACTTCYGGGTGRCCRAARAATCA-3') (Baldwin et al. 2009), 2 mM $MgCl_2$, and 0.25 U of Platinum™ SuperFi II DNA Polymerase. Both primers contained five additional bases serving as barcodes for sequencing. The final reaction volume was adjusted with sterile ultrapure water. PCR amplification was carried out under the following cycling conditions: initial denaturation at 94 °C for 2 min; 35 cycles of 94 °C for 20 s, 54 °C for 35 s, and 72 °C for 35 s; and a final extension at 72 °C for 2 min followed by a hold at 40 °C.

PCR products were subjected to electrophoresis on a 1% agarose gel in 1× TAE buffer. A sequencing library was constructed using the Ligation Sequencing Kit SQK-LSK114 (Oxford Nanopore, UK), following the NEBNext protocol (New England Biolabs, UK). This step involved end-repair of the amplified sequences and adapter ligation. The library was loaded onto a Flongle Flow Cell (FLO-FLG114) and sequenced using a MinION Mk1B device with a Flongle Adapter (ADP-FLG001) managed by MinKNOW v. 24.06.08 software (Oxford Nanopore Technologies, UK). Data were processed using Super Accurate Basecalling mode; only sequences with a minimum quality score of 8 were retained. A custom pipeline clustered similar sequences to extract a representative consensus.

Phylogenetic tree construction used COI sequences of *H. multifasciatus* from Thomaz et al. (2010), with two *Rachoviscus* sequences as outgroups. All analyses were conducted in the R environment, based on a prior alignment of the sequences (FASTA format), using the packages *ape* (Paradis and Schliep 2019) and *phangorn* (Schliep 2011). The initial tree was constructed via Neighbor-Joining method and refined by Maximum Likelihood (ML), using the GTR+I model selected via Akaike Information Criterion (AIC). The tree was optimized, and branch support was assessed through 1,000 bootstrap replicates, with only support values above 50% represented in the final tree. Additionally, a haplotype network was constructed using the TCS method (Clement et al. 2000), in PopArt v.1.7 (Leigh and Bryant 2015).

Sequencing results show that *H. multifasciatus* from the upper rio Iguaçu are closely related to populations from coastal basins draining into Paranaguá Bay, which is an expected result since these represent the geographically closest populations (Fig. 1). This region is influenced by the Serra do Mar Rift System, the largest structural discontinuities of the South American Platform (Ribeiro 2006). The occurrence of the species in the upper Iguaçu is likely associated with historical headwater capture events between neighboring basins of the upper Rio Iguaçu and coastal streams, promoting faunal exchange and the expansion of distribution among adjacent drainages (Bishop 1995). This indicates that the upper Rio Iguaçu has recently received fish species once restricted to coastal rivers, a pattern recognized as "Pattern C" (Ribeiro 2006), consistent with records of *Hyphessobrycon griemi* Hoedeman, 1957, *H. bifasciatus* Ellis, 1911, *Mimagoniates microlepis* (Steindachner, 1877), *Gymnotus pantherinus*

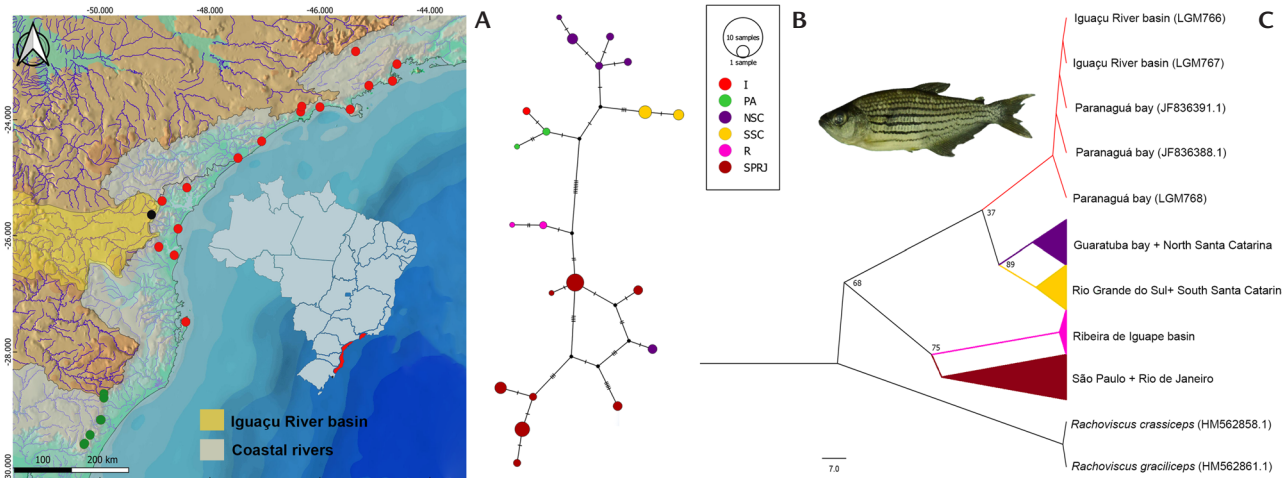

Figure 1. Geographic distribution, haplotype network, and phylogenetic relationships of *Hollandichthys multifasciatus*. (A) Geographic distribution based on records from Thomaz et al. (2010) and the new record from the upper Rio Iguaçu basin. Red circles: localities identified as *H. multifasciatus* by Thomaz et al. (2010); green circles: records from southern Santa Catarina and Rio Grande do Sul currently recognized as *H. taramandahy*; black circle: new record from the upper Rio Iguaçu basin. Shaded areas represent the Rio Iguaçu basin (yellow) and adjacent coastal drainages (light blue). (B) Haplotype network (TCS method) of *H. multifasciatus* populations based on mitochondrial COI sequences. Circle size is proportional to haplotype frequency. Geographic regions: I – upper Rio Iguaçu; PA – Paranaguá Bay; NSC – Guaratuba Bay and northern Santa Catarina; SSC – southern Santa Catarina and Rio Grande do Sul; R – Ribeira de Iguape basin; SPRJ – São Paulo and Rio de Janeiro. (C) Maximum Likelihood phylogenetic tree. Numbers at nodes indicate bootstrap support values.

(Steindachner, 1908), and *Phalloceros titthos* Lucinda, 2008 in the drainage (Ingenito et al. 2004, Souto-Santos et al. 2025).

The presence of *H. multifasciatus* in the upper Rio Iguaçu represents the second known record of the genus in a non-coastal basin, following the record for the upper Rio Tietê (Marceniuk et al. 2011). This region, on the western side of the Serra do Mar, presents significant geological and biological evidence of headwater capture. A well-documented case involves the upper course of the Rio Guaratuba, which originally drained inland toward the upper Rio Paraná basin; however, following Quaternary tectonic reactivations, its course was redirected to the coast (Ribeiro et al. 2006). This example illustrates the role of headwater capture as a key process in the dispersal and diversification of the Neotropical ichthyofauna. Nevertheless, the mechanisms responsible for the occurrence of coastal fish faunas in inland drainages, such as the upper rio Iguaçu, remain poorly understood (Tagliacollo et al. 2015, Lavarini et al. 2016).

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Competing Interests

The authors have declared that no competing interests exist.

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