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PENSOFT

Geographic distribution of *Phalloceros* Eigenmann, 1907 (Cyprinodontiformes, Poeciliidae) in the Ilha Grande Bay Hydrographic Region, Rio de Janeiro, Brazil

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Abstract

The diversity and geographic distribution of *Phalloceros* Eigenmann, 1907 in the Ilha Grande Bay Hydrographic Region, in southeastern Brazil, is investigated. Examination of 81 samples revealed the presence of 5 species in the region: *Phalloceros anisophallos* Lucinda, 2008, *P. aspilos* Lucinda, 2008, *P. enneaktinos* Lucinda, 2008, *P. harpagos* Lucinda, 2008, and *P. tupinamba* Lucinda, 2008. *Phalloceros harpagos* and *P. tupinamba* are recorded for the first time in the area. The geographic ranges of *P. anisophallos* and *P. enneaktinos* are expanded. The 5 species belong to 3 distinct morphologies of the female urogenital papilla. Species from these distinct morphological lineages may occur sympatrically, but species within the same lineage are always allopatric. A key for the identification of these species is provided.

Key words

Angra dos Reis, fish diversity, livebearers, molecular diversity, Parati, poeciliids; taxonomy.

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Introduction

Phalloceros Eigenmann, 1907 is a monophyletic genus of small livebearing fishes distributed in freshwaters of southeastern South America, occurring between the southern portion of the Brazilian state of Bahia and Uruguay, as well as in the western portion of the La Plata river system in Argentina and Paraguay, and the rio Araguaia drainage (Lucinda and Reis 2005, Lucinda 2008). The genus is diagnosed by (1) the presence of a pair of appendices in the tip of the third ray of the gonopodium, and (2) the presence of partially closed preopercular canal (Lucinda 2008). For more than a century, *Phalloceros* was treated as a monotypic genus, with *Phalloceros caudimaculatus* (Hensel 1868) regarded as a single widely distributed species. In 2005, however, a phylogenetic analysis of the Poeciliidae recognized 21 additional *Phalloceros* species (Lucinda and Reis 2005), which were formally described 3 years later (Lucinda 2008).

Several species of *Phalloceros* are geographically restricted to coastal drainages in southeastern Brazil, but the exact limits of their distributions are still poorly known due to limited sampling. Such is the case of the river drainages located in Parati (= Paraty) and Angra

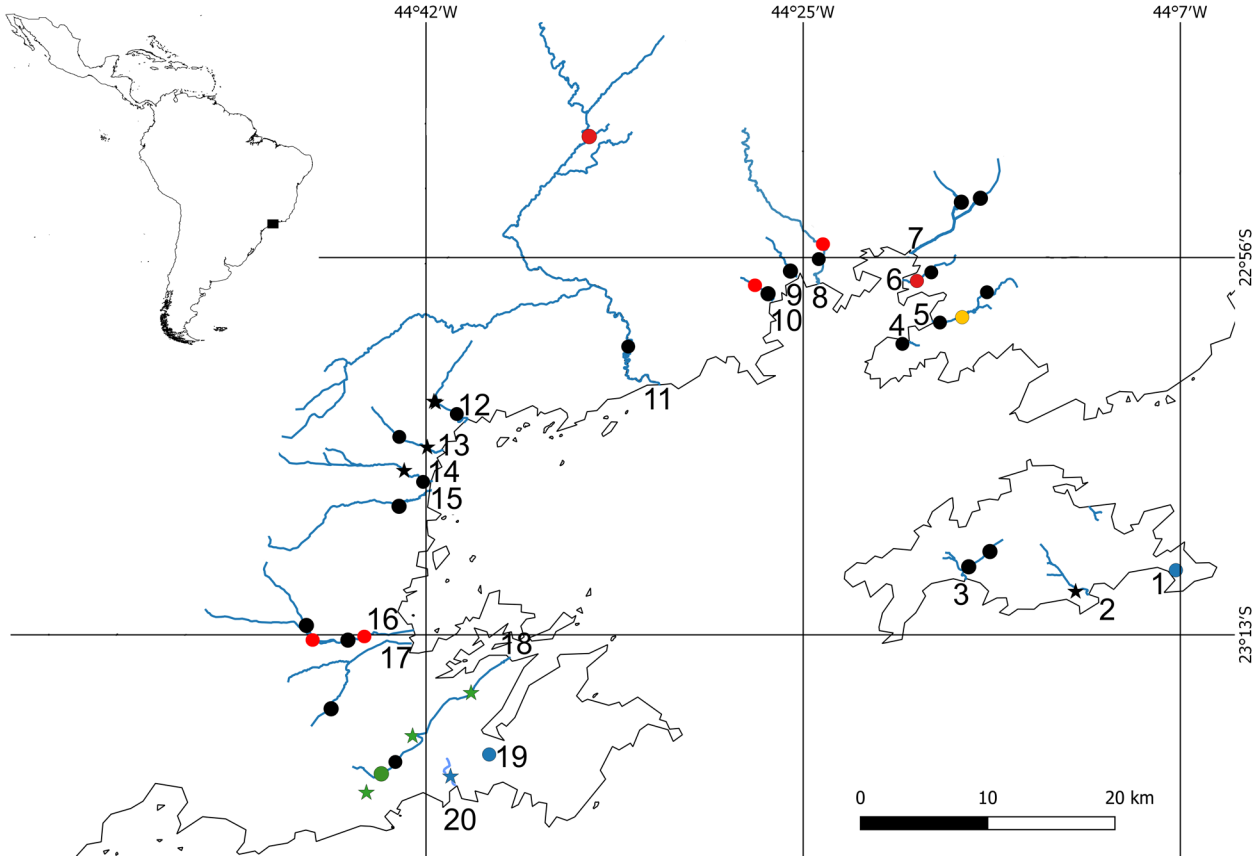

Figure 1. Geographic distribution of species of *Phalloceros* in the Ilha Grande Bay Hydrographic Region. River drainages are numbered according to Table 1. Black: *P. anisophallos*. Green: *P. aspilos*. Blue: *P. enneaktinos*. Red: *P. harpagos*. Yellow: *P. tupinamba*. Stars represent localities previously recorded by Lucinda (2008). Circles represent new records. Symbols may represent more than one sample.

dos Reis municipalities, most of which flow into the Ilha Grande Bay on the western coast of the Rio de Janeiro state, hereafter referred to as Ilha Grande Bay Hydrographic Region (SEMADS 2001). Three species of *Phalloceros* were previously recorded in the area based on limited samples: (1) *Phalloceros aspilos* Lucinda, 2008, described from the rio Parati Mirim drainage; (2) *Phalloceros enneaktinos* Lucinda, 2008, described from the rio Toca do Boi drainage; (3) and *Phalloceros anisophallos* Lucinda, 2008, recorded from the rio Taquari, rio São Roque, rio Barra Grande and rio Andorinhas drainages (Lucinda 2008). Here we report the presence of 2 additional species in this hydrographic region and significantly expand the known distribution of the previously reported species. A key for identification of *Phalloceros* species in this area is also provided.

Methods

Study site. The study area comprises the IGBHR (Fig. 1), which includes both mainland and island catchment areas that drain into the Ilha Grande bay, including small streams located in the municipalities of Parati and Angra dos Reis that flow into the Atlantic Ocean in southeastern Brazil (SEMADS 2001). These continental drainages extend from the mouth of the rio Jacareí (23°02′01″S, 044°09′56″W) in the east to the western border of the Rio de Janeiro state where it meets the ocean (23°22′06″S, 044°43′27″W). Additionally, the largest island inside the bay, Ilha Grande, includes several small freshwater drainages, some of which support stable freshwater fish populations. The adjacent mainland rivers drain the coastal lowlands and the slopes of the Serra do Mar while the headwaters of some of these rivers drain the main plateau of the Bocaina highlands (Francisco and Oliveira 2009).

Collection. The present study is based on examination of specimens collected between 1942 and 2017 (Table 1). These specimens are housed at the Ichthyological Collection of the Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ). Species identification was based on morphological characters of sexually mature specimens, according to Lucinda (2008). The identification of juvenile specimens from sites where more than 1 species occurs sympatrically was based on comparisons of pigmentation and position of the lateral spot. Osteological characters were observed in cleared and stained specimens (Taylor and Van Dyke 1985) and digital radiographs.

The distribution map of the examined material (Fig. 1) was generated with Quantum GIS (QGIS Development Team 2017), using river traces obtained from Google Earth satellite images. In addition to MNRJ specimens, the map includes georeferenced records listed by Lucinda (2008).

Table 1. River drainages sampled in the Ilha Grande Bay Hydrographic Region, numbered from east to west according to position of their outlet into the sea (Fig. 1). Drainages 1 to 3 are located on Ilha Grande.

Number	River drainage
1	Praia de Lopes Mendes
2	Córrego Andorinha
3	Sistema Lagunar do sul e Leste
4	Praia do Retiro
5	Rio Japuíba (=Rio Japuhyba or Rio do Meio)
6	Rio Caputera
7	Rio Jurumirim
8	Rio Bracuí
9	Rio do Saco
10	Rio Ambrósio
11	Rio Mambucaba
12	Rio Taquari
13	Rio São Roque
14	Rio Barra Grande
15	Rio Pequeno
16	Rio Perequê Açu
17	Rio Matheus Nunes
18	Rio Parati-Mirim
19	Saco do Mamanguá
20	Córrego Toca do Boi

DNA barcoding and species delimitation. During this study, variation in the number of dorsal fin-ray counts was detected in *P. ennektinos*, limiting the usefulness of morphological characters to distinguish that species from *P. harpagos* Lucinda 2008. DNA barcoding methods (Hebert et al. 2003a) were then used to differentiate these 2 species. Muscular tissue from specimens preserved in anhydrous ethanol were processed for DNA extraction following the salting out method (Miller et al. 1988). DNA quality was verified with standard agarose gel electrophoresis, and DNA concentration was measured with a NanoDrop ND-2000 spectrophotometer. Partial sequences of the Cytochrome Oxidase Subunit I (COI) gene were amplified with Polymerase Chain Reaction (PCR) using primers Fish-F6 (M13f -21) 5′-TGT AAA ACG ACG GCC AGT ACY AAY CAC AAA GAY ATT GGC A-3′ and Fish-R7 (M13r-29) 5′-CAG GAA ACA GCT ATG ACC TAR ACT TCT GGR TGD CCR AAG AAY CA-3′ which included M13 sequencing primer sequences (underlined) appended (Jennings et al. in press). The PCR protocol used the following thermal cycle: denaturation at 94 °C/30 s, primer hybridization at 50 °C/45 s, and nucleotide extension at 72 °C/45 s; this sequence was repeated 35 times in a loop, and the reaction was terminated with 72 °C/10 min and 4 °C/5 min. Quality of amplified products was verified with 2% agarose-gel electrophoresis. PCR products were purified using PEG (Lis 1980, Jennings 2016), and bidirectionally sequenced with the Sanger method, where a BigDyeTM Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems) was used for labeling, purified again with ethanol precipitation and loaded on an automatic sequencer ABI3730xl (Applied Biosystems) at Fundação Oswaldo Cruz. The resulting chromatograms were aligned to a reference sequence using the Geneious R9.1 software and manually edited to ensure codon alignment and to verify base calls. All sequences and associated data, including geospatial coordinates, sequence data, trace files, and primer details are available in the Barcode of Life Data Systems (BOLD Systems, http://www.boldsystems.org/; Ratnasingham and Hebert 2007). Sample details are listed in Table 2, along with GenBank Accession codes. Levels of genetic divergence among samples was evaluated using Refined Single Linkage Analysis (Ratnasingham and Hebert 2013) available through BOLD Systems. Specimens with genetic distances less than 2% were considered conspecific following DNA barcoding standards for fish (Hebert el al. 2003a, 2003b, Pereira et al. 2013). A

Table 2. List of specimens used to obtain molecular data in this study. Sample ID refers to the DNA Extract Collection of the Laboratório de Pesquisa em Biodiversidade Molecular, Museu Nacional (MNLM) which are also used to record specimen data in BoldSystems (http://www.boldsystems.org/). Voucher code refers to the whole-organism catalog numbers, and specimen/tissue code refers to tissue catalog number.

Species	Sample ID	Voucher code	Specimen/ tissue code	BoldSystems process ID	GenBank accession
P. enneaktinos	MNLM6692	MNRJ43245	MNTI10545	MNRJ1028-18	MH570255
P. enneaktinos	MNLM6693	MNRJ43245	MNTI10546	MNRJ1029-18	MH570251
P. enneaktinos	MNLM6794	MNRJ43245	MNTI10547	MNRJ1030-18	MH570258
P. enneaktinos	MNLM6795	MNRJ43245	MNTI10548	MNRJ1031-18	MH570261
P. enneaktinos	MNLM7068	MNRJ42910	MNTI10976	MNRJ1038-18	MH570253
P. enneaktinos	MNLM7069	MNRJ42910	MNTI10977	MNRJ1039-18	MH570259
P. enneaktinos	MNLM7070	MNRJ42910	MNTI10978	MNRJ1040-18	MH570257
P. enneaktinos	MNLM7071	MNRJ42910	MNTI10979	MNRJ1041-18	MH570263
P. enneaktinos	MNLM7084	MNRJ42910	MNTI10980	MNRJ1042-18	MH570266
P. enneaktinos	MNLM7085	MNRJ42910	MNTI10981	MNRJ1043-18	MH570264
P. harpagos	MNLM6992	MNRJ43508	MNTI10829	MNRJ1032-18	MH570256
P. harpagos	MNLM6993	MNRJ43508	MNTI10830	MNRJ1033-18	MH570262
P. harpagos	MNLM6994	MNRJ43508	MNTI10831	MNRJ1034-18	MH570260
P. harpagos	MNLM6995	MNRJ43508	MNTI10832	MNRJ1035-18	MH570265
P. harpagos	MNLM6996	MNRJ43508	MNTI10833	MNRJ1036-18	MH570252
P. harpagos	MNLM7031	MNRJ43508	MNTI10834	MNRJ1037-18	MH570254

haplotype network was inferred using the TCS method on PopART (Leigh and Bryant 2015).

Results

Examination of 81 samples comprising 3,386 specimens, which were collected since 1942 in 20 river drainages (Table 1), allowed us to recognize the presence of 5 species of *Phalloceros* in the IGBHR: *Phalloceros anisophallos*, *P. aspilos*, *P. enneaktinos*, *P. harpagos*, and *P. tupinamba* (Fig. 2). These new distribution records in addition to issues involving species identifications are discussed below.

***Phalloceros anisophallos* Lucinda, 2008**
Figure 2A

Materials examined. Brazil: Rio de Janeiro: Córrego Andorinha, Ilha Grande, município de Angra dos Reis (23°11′12″ S, 044°12′02″ W), R. Mazzoni, C.F. Rezende, 4 February 2002 (MNRJ28725). Canal principal do manguezal das lagunas do Sul e Leste, 200 m da laguna do Leste, município de Angra dos Reis (23°10′ S, 044°17′ W), J.R. Gomes, 2 January 2005 (MNRJ30524). Córrego do Cardoso, contribuinte do sistema lagunar do Sul e Leste, extremo oeste da laguna do Sul, Ilha Grande, município de Angra dos Reis (23°10′ S, 044°18′ W), J.R. Gomes, 6 January 2005 (MNRJ30540). Rio Capivari, montante da laguna do Leste, município de Angra dos Reis (23°09′ S, 044°16′ W), J.R. Gomes, A.T. Aranda, E. Chaves, G.D. Estrada, A. Cardoso, 10 September 2005 (MNRJ30675). Pequeno rio a montante da Praia do Retiro, Estrada do Contorno, município de Angra dos Reis, J.R. Gomes, 01 August 2003 (MNRJ26465). Rio Japuíba, Fazenda Japuíba, município de Angra dos Reis, H. Travassos, 12 September 1944 (MNRJ5960). Rio Japuíba, Fazenda Japuíba, município de Angra dos Reis, L. Travassos, H. Travassos, E. Travassos, 28 April 1945 (MNRJ42357). Rio Japuíba, Fazenda Japuíba, município de Angra dos Reis, P. Miranda-Ribeiro, G.S. Myers, H. Travassos, August 1942 (MNRJ42915, MNRJ42916). Córrego Roncador, tributário do rio Japuíba na estrada de Banqueta. Município de Angra dos Reis (22°57′24″ S, 044°16′07″ W), P.A. Buckup, K.C. Oliveira, C.E. Lopes, I. Rubio, 12 April 2008 (MNRJ32678). Rio Roncador, drenagem do rio Japuíba, Município de Angra dos Reis (22°57′23″ S, 044°16′6″ W), P.A. Buckup, D.F. Moraes Jr, I.C.A. Souto-Santos, M.M. Gonzalez, D.R.S. Peixoto, J.R. Amaral, 12 October 2014 (MNRJ43520). Rio Caputera, 9.2 Km (em linha reta) a montante da ponte da rodovia Rio-Santos (BR-101), município de Angra dos Reis (22°56′29″ S, 044°18′41.1″ W), P.A. Buckup, D.F. Moraes Jr, I.C.A.Souto-Santos, M.M. Gonzalez, D.R.S. Peixoto, J.R. Amaral, 12 October 2014 (MNRJ43507). Rio Caputera ou Areia do Pontal, 2 km a montante da ponte sobre o mesmo rio (rodovia Rio-Santos, BR-101), Município de Angra dos Reis, D.F. Belote, 5 February 2003 (MNRJ24245). Rio Serra D'Água, em Serra D'água, Município de Angra dos Reis (22°53′18″ S, 044°16′18″ W), P.A. Buckup, M.R. Britto, F.L.K. Salgado, L. Villa-Verde, J.R. Gomes, P. Petry, J. Quartim, 13 September 2010 (MNRJ38070). Rio Zungu, município de Angra dos Reis (22°53′14″ S, 044°17′18″ W), P.A. Buckup, M.R. Britto, F.L.K. Salgado, L. Villa-Verde, J.R. Gomes, P. Petry, J. Quartim, 13 September 2010 (MNRJ38083). Rio Zungu e tributário, a montante da ponte da estrada de terra, município de Angra dos Reis (22°53′14″ S, 044° 17′18″ W), P.A. Buckup, K.C. Oliveira, C.E. Lopes, I. Rubio, 12 April 2008 (MNRJ32688, MNRJ32689). Rio Bracuí, Frade, município de Angra dos Reis, Rodovia Rio-Santos (BR-101) Km 115, O. Pereira, H. Barros, 28 August 1983 (MNRJ22142). Rio Bracuí, junto ao Bar da Cachoeira, Quilombo Santa Rita I, município de Angra dos Reis (22°54′34″ S, 044°24′31″ W), P.A. Buckup, D.F. Moraes Jr, V. de Brito, L. Villa-Verde, G. Beltrão, 3 July 2015 (MNRJ43851). Segundo afluente do rio Bracuí, município de Angra dos Reis, H. São-Thiago, D.A. Halboth, J.H.C. Gomes, E.P. Caramaschi, 1 July 1988 (MNRJ47041). Rio do Saco, junto a rodovia Rio-Santos (BR-101), município de Angra dos Reis (22°56′25″ S, 044°25′11″ W), P.A. Buckup, D.F. Moraes Jr, V. de Brito, L. Villa-Verde, G. Beltrão, 3 July 2015 (MNRJ43859). Rio Ambrósio, a jusante da ponte da rodovia Rio-Santos (BR-101), em Frade (= Cunhambebe), município de Angra dos Reis (22°57′28″ S, 044°26′13″ W), P.A. Buckup, D.F. Moraes Jr, V. de Brito, L. VillaVerde, G. Beltrão, 3 July 2015 (MNRJ43897). Rio Mambucaba, município de Angra dos Reis, M. Vianna, J.M.R. Aranha, J.H.C. Gomes, 11 January 1989 (MNRJ11597). Afluente do rio Taquari, município de Angra dos Reis (23°02′29″ S, 044°41′38″ W), B. Gorini-Pacheco, 23 January 2017 (MNRJ50836). Rio Taquari, município de Angra dos Reis (23°02′47″ S, 044°41′5″ W), B. Gorini-Pacheco, 27 January 2017 (MNRJ50839). Rio São Roque a montante da ponte da rodovia Rio-Santos (BR-101) (23°04′33.3″ S, 044°41′56.5″ W), P.A. Buckup, D.F. Moraes Jr, I.C.A. Souto-Santos, M.M. Gonzalez, D.R.S. Peixoto, J.R. Amaral, 10 October 2014 (MNRJ43253). Afluente do rio São Roque, município de Angra dos Reis (23°04′04″ S, 044°43′14″ W), B. Gorini-Pacheco, 25 January 2017 (MNRJ50837). Rio São Roque, município de Angra dos Reis (23°04′35″ S, 044°41′51″ W), B. Gorini-Pacheco, 24 January 2017 (MNRJ50838). Trecho superior do rio Barra Grande, município de Parati, F.J. Lobón-Cerviá, 5 November 1993 (MNRJ12502). Rio Barra Grande, município de Angra dos Reis (23°05′33″ S, 044°43′05″ W), B. Gorini-Pacheco, 26 January 2017 (MNRJ50840). Rio Pequeno, rodovia Rio-Santos (BR-101), município de Parati, D.F. Moraes Jr, et al., 19 July 1987 (MNRJ49130). Rio Perequê Açu, bairro Ponte Branca, estrada Parati-Cunha, Km 3,5, nos fundos do restaurante Lingo-Lingo, município de Parati (23°13′27″ S, 044°45′36″ W), R. Souza-Lima, R.R. Rodrigues Jr, F.V. Guimarães, T.M. Souza, 24 June 2013 (MNRJ41625). Rio Perequê Açu, bairro Ponte Branca, estrada Parati-Cunha – Km 3,5, nos fundos do restaurante Lingo-Lingo, município de Parati (23°13′24″ S, 044°45′30″ W),

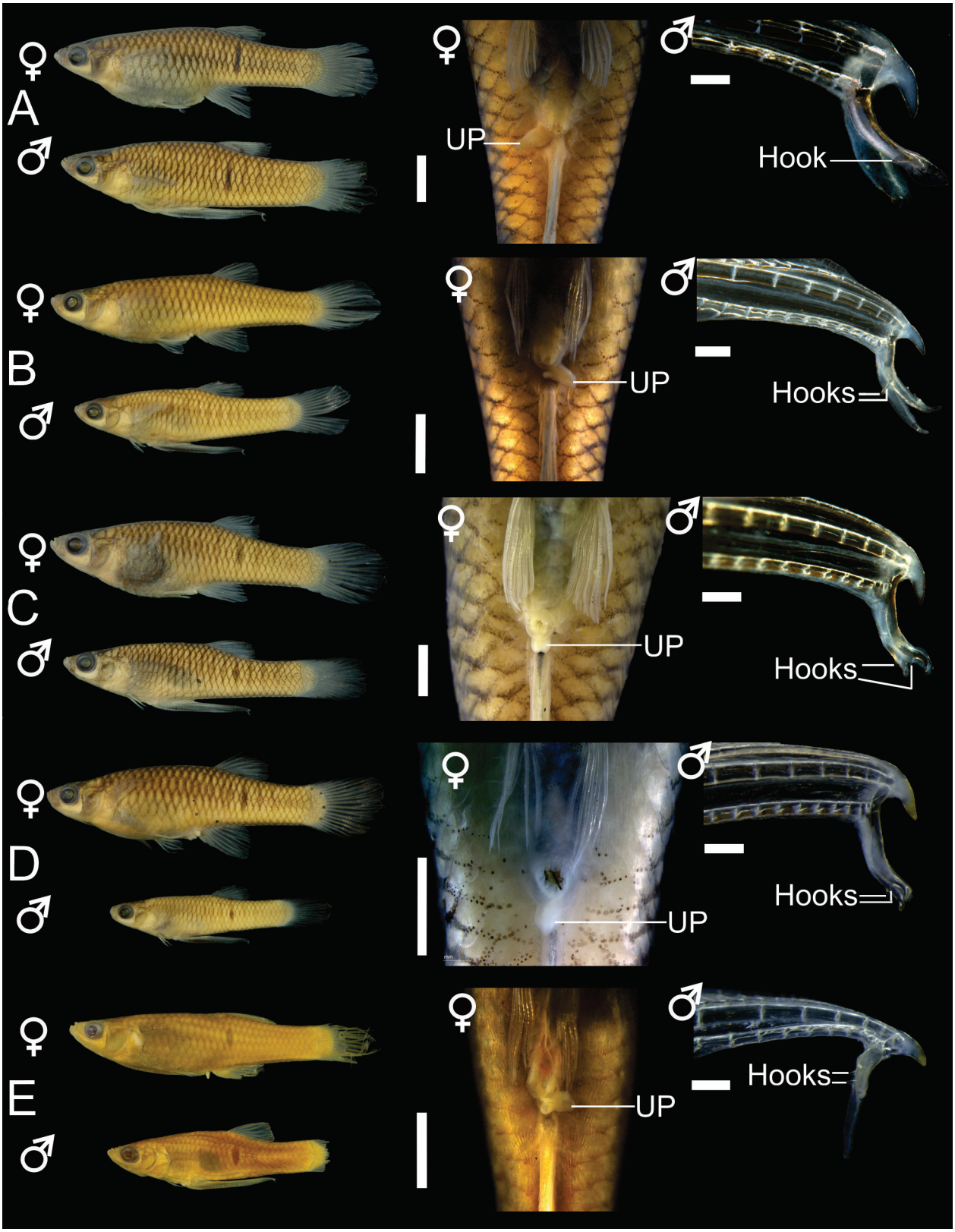

Figure 2. Species of *Phalloceros* from IGBHR with close-ups of female urogenital papillae (UP) and tip of gonopodia. **A.** *Phalloceros anisophallos*, lateral view (MNRJ43253, ♀ 34.8 mm SL, ♂ 26.0 mm SL), ventral view (♀ MNRJ43253), tip of gonopodium (♂ MNRJ43598). **B.** *Phalloceros aspilos*, lateral view (MNRJ43505, ♀ 31.3 mm SL, ♂ 20.9 mm SL). **C.** *Phalloceros enneaktinos*, lateral view (MNRJ43245, ♀ 24.9 mm SL, ♂ 22.9 mm SL), ventral view (♀ MNRJ20252), tip of gonopodium (♂ MNRJ20252). **D.** *Phalloceros harpagos*, lateral view (MNRJ43508, ♀ 36.2 mm SL, ♂ 19.5 mm SL), ventral view (♀ MNRJ43508), tip of gonopodium (♂ MNRJ43508). **E.** *Phalloceros tupinamba*, lateral view (MNRJ4226, ♀ 32.0 mm SL, ♂ 21.2 mm SL), ventral view (♀ MNRJ8548), tip of gonopodium (♂ MNRJ8518). Vertical scale bars are 2 mm; horizontal scale bars are 0.2 mm.

R. Souza-Lima, R.R. Rodrigues Jr, F.V. Guimarães, T.M. Souza, 30 September 2013 (MNRJ42084). Rio Carrasquinho, poço imediatamente a jusante da Cachoeira do Tobogã no complexo turístico do bairro da estrada Parati-Cunha, aproximadamente Km 13, município de Parati (23°12′46″ S, 044°47′31″ W), R. Souza-Lima, R.R. Rodrigues Jr, F.V. Guimarães, T.M. Souza, 26 June 2013 (MNRJ41639). Rio Perequê Açu, nas proximidades da rua A, entrada antes da loja "Trevo-Materiais de Construção" na estrada Parati-Cunha a montante da última ponte antes do bairro do Condado, antes da rodovia Rio-Santos (BR-101), município de Parati (23°13′23″ S, 044°45′4″ W), R. Souza-Lima, R.R. Rodrigues Jr, F.V. Guimarães, T.M. Souza, 3 October 2013 (MNRJ42089). Córrego da Laje, afluente da margem direita do rio Pedra Branca, próximo a sua foz. Bairro da Ponte Branca, Km 1 da estrada Parati, município de Parati (23°13′27″ S, 044°45′36″ W), R. Souza-Lima, R.R. Rodrigues Jr, F.V. Guimarães, T.M. Souza, 28 June 2013 (MNRJ41642). Rio dos Sertões, entrando pelo sítio e restaurante Engenho D'Ouro, Penha, município de Parati (23°13′26″ S, 044°45′35″ W), R. Souza-Lima, et al., 25 August 2015 (MNRJ50622). Rio Corisco, a montante de Corisquinho, junto a ponte abandonada, município de Parati (23°16′24″ S, 044°46′21″ W), P.A. Buckup, R. Souza-Lima, 19 February 2016 (MNRJ45003). Rio Parati-Mirim, imediatamente a montante da ponte da vila do Patrimônio, município de Parati (23°19′03.7″ S, 044°43′24.7″ W), P.A. Buckup, D.F. Moraes Jr, I.C.A. Souto-Santos, M.M. Gonzalez, D.R.S. Peixoto, J.R. Amaral, 10 October 2014 (MNRJ43504). Rio Parati-Mirim, Km 4 da estrada de terra que liga a BR-101 a vila Parati-Mirim, município de Parati, H. São-Thiago, D.F. Moraes Jr, 18 December 1987 (MNRJ43117). Rio Parati-Mirim, Km 4 da estrada de terra que liga a BR-101 a vila Parati-Mirim, município de Parati, H. São-Thiago, D.F. Moraes Jr, 28 August 1987 (MNRJ43121). Rio Parati-Mirim, Km 4 da estrada de terra que liga a BR-101 a vila Parati-Mirim, município de Parati, H. São-Thiago, D.F. Moraes Jr, W.D. Bandeira, 28 January 1989 (MNRJ43127). Rio Parati-Mirim, Km 4 da estrada de terra que liga a BR-101 a vila Parati-Mirim, município de Parati, H. São-Thiago, D.F. Moraes Jr, 10 April 1989 (MNRJ43129).

Identification. See taxonomic key below.

Distribution. In the IGBHR, *P. anisophalos* was previously recorded from the rio Taquari, rio São Roque, and rio Barra Grande drainages in the mainland, and from the rio Andorinhas drainage in Ilha Grande (Lucinda 2008). This species was also known from the rio Itinguçu drainage, which is located to the east of the IGBHR and flows into the Sepetiba Bay (Lucinda 2008). The known distribution of this species in the IGBHR is expanded here to the following mainland drainages: rio Jurumirim, rio Caputera, rio Japuíba, Praia do Retiro, rio Bracuí, rio do Saco, rio Ambrósio, rio Mambucaba, rio Pequeno, rio Perequê Açu, rio Mateus Nunes, and rio Parati Mirim. New records from Ilha Grande, include the rio Cardoso and rio Capivari, both of which flow into the main channel of the mangroves of Sul and Leste lagoons. *Phalloceros anisophallos* is the most geographically widespread species of *Phalloceros* in the IGBHR, having been recorded in 17 of the 20 sampled river drainages (Fig. 1). In four of these drainages it was found in sympatry with other species of *Phalloceros*: *P. harpagos* in the rio Bracuí and rio Caputera, *P. tupinamba* in the rio Japuíba, and *P. aspilos* in the rio Parati Mirim (Fig. 1).

***Phalloceros aspilos* Lucinda, 2008**
Figure 2B

Materials examined. Brazil: Rio de Janeiro: Rio Parati-Mirim, imediatamente a montante da ponte da Vila do Patrimônio, município de Parati (23°19′03.7″ S, 044°43′ 24.7″ W), P.A. Buckup, D.F. Moraes Jr, I.C.A. Souto-Santos, M.M. Gonzalez, D.R.S. Peixoto, J.R. Amaral, 10 October 2014 (MNRJ43505). Rio Parati-Mirim, Km 202 da rodovia Rio-Santos (BR-101), à montante da Vila do Patrimônio, município de Parati, H. São-Thiago, D.F. Moraes Jr, W.D. Bandeira, 29 January 1989 (MNRJ11727, MNRJ23607). Afluente da margem esquerda do rio "Forquilha" (afluente do rio Parati-Mirim), na estrada que vai de Patrimônio para Forquilha, município de Parati, no collector data, 03 March 1989 (MNRJ43115). Rio Parati-Mirim, Km 4 da estrada de terra que liga a BR-101 a vila Parati-Mirim, município de Parati, H. São-Thiago, D.F. Moraes Jr, W.D. Bandeira, 28 January 1989 (MNRJ43118). Rio Parati-Mirim, Km 4 da estrada de terra que liga a BR-101 a vila Parati-Mirim, município de Parati, H. São-Thiago, D.F. Moraes Jr, 28 August 1987 (MNRJ43123). Rio Parati-Mirim, Km 4 da estrada de terra que liga a BR-101 a vila Parati-Mirim, município de Parati, H. São-Thiago, D.F. Moraes Jr, 10 April 1989 (MNRJ43128). Rio Parati-Mirim, km 202 da rodovia Rio-Santos (BR-101), em Vila do Patrimônio, município de Parati, H. São-Thiago, D.F. Moraes Jr, 15 July 1987 (MNRJ43119). Rio Parati-Mirim, km 202 da rodovia Rio-Santos (BR-101), em Vila do Patrimônio, município de Parati, H. São-Thiago, D.F. Moraes Jr, 17 December 1987 (MNRJ43120). Rio Parati-Mirim, km 202 da rodovia Rio-Santos (BR-101), em Vila do Patrimônio, município de Parati, H. São-Thiago, D.F. Moraes Jr, June 1988 (MNRJ43124). Rio Parati-Mirim, km 202 da rodovia Rio-Santos (BR-101), em Vila do Patrimônio, município de Parati, H. São-Thiago, D.F. Moraes Jr, 8 October 1988 (MNRJ43125). Rio Parati-Mirim, km 202 da rodovia Rio-Santos (BR-101), em Vila do Patrimônio, município de Parati, H. São-Thiago, D.F. Moraes Jr, 11 April 1989 (MNRJ43126). Rio Parati-Mirim, a montante da rodovia Rio-Santos (BR-101), município de Parati, H. São-Thiago, D.F. Moraes Jr, 28 Janaury 1989 (MNRJ43130). Rio Parati-Mirim, próximo e a montante da ponte da rodovia Rio-Santos (BR-101), município de Parati, H. São-Thiago, D.F. Moraes Jr, 10 April 1989 (MNRJ43131). Rio Parati-Mirim, a montante da ponte da rodovia Rio-Santos (BR-101), município de Parati, H. São-Thiago,

D.F. Moraes Jr, 10 December 1988 (MNRJ43132). Rio Parati-Mirim, a montante da ponte da rodovia Rio-Santos (BR-101), município de Parati, H. São-Thiago, D.F. Moraes Jr, 14 August 1988 (MNRJ43133). Rio Parati-Mirim, a montante da ponte da rodovia Rio-Santos (BR-101), município de Parati, H. São-Thiago, D.F. Moraes Jr, 4 June 1988 (MNRJ43134). Rio Parati-Mirim, a montante da ponte da rodovia Rio-Santos (BR-101), município de Parati, H. São-Thiago, D.F. Moraes Jr, 18 December 1987 (MNRJ43136). Rio Parati-Mirim, a montante da ponte da rodovia Rio-Santos (BR-101), município de Parati, H. São-Thiago, D.F. Moraes Jr, 28 August 1987 (MNRJ43137). Rio Parati-Mirim, Km 202 da rodovia Rio Santos (BR-101), a montante da Vila do Patrimônio, município de Parati, H. São-Thiago, D.F. Moraes Jr, 03 April 1988 (MNRJ43135). Afluente do rio Parati-Mirim cruzando a estrada que liga a cidade de Parati-Mirim a rodovia Rio-Santos (BR-101), município de Parati, O. Pereira, H. Barros, 27 August 1983 (MNRJ41429).

Identification. *Phalloceros aspilos* was originally diagnosed by a unique autapomorphic trait of the caudal skeleton, which has been described as "hypural plate almost bipartite, with very large aperture" (Lucinda 2008, character state 131-3 in Lucinda and Reis 2005). However, our examination of the caudal skeleton of 32 specimens (14 males, 16 females) revealed that the configuration of the hypural plate varies in *P. aspilos*. The state described by Lucinda and Reis (2005) was observed in only 2 males of our sample while the remaining specimens had a condition in which the hypural aperture is longer, almost eliminating the distal bony bridge connecting the 2 portions of the hypural plate (described as "hypural plate partially fused with an elongate aperture" character state 131-1 in Lucinda and Reis 2005) (Fig. 3). Nevertheless, *P. aspilos* may be distinguished from other species of *Phalloceros*, except *P. tupinamba* and *P. leptokeras* Lucinda, 2008, by the leftward orientation of the female urogenital papilla. From *P. tupinamba* and *P. leptokeras*, *P. aspilos* is distinguished by the lack of the lateral black spot.

Distribution. This species is restricted to the rio Parati Mirim drainage, in the western portion of the IGBHR (Fig. 1), as reported by Lucinda (2008).

Phalloceros enneaktinos Lucinda, 2008

Figure 2C

Materials examined. Brazil: Rio de Janeiro: Área alagada na restinga da praia de Lopes Mendes, abastecida por um riacho, Ilha Grande, município de Angra dos Reis (23°10′13″ S, 044°07′24″ W), R.M. Dias, N. Nagatani, 9 November 2014 (MNRJ42910). Charco na trilha de Mamanguá, município de Parati (23° 18′43″ S, 044°39′05″ W), M.R.S. Melo, P.A. Buckup, 21 April 2000 (MNRJ20250). Córrego da Toca do Boi, próximo ao portão de guarda do condomínio Laranjeiras, município de Parati (23°19′44″ S, 044°40′52″ W), M.R.S. Melo, P.A. Buckup, 23 April 2000 (MNRJ20252). Córrego da Toca do Boi, próximo a ponte a montante do condomínio Laranjeiras, município de Parati (23°19′44.8″ S, 044°40′54.6″ W), P.A. Buckup, D.F. Moraes Jr, I.C.A. Souto-Santos, M.M. Gonzalez, D.R.S.

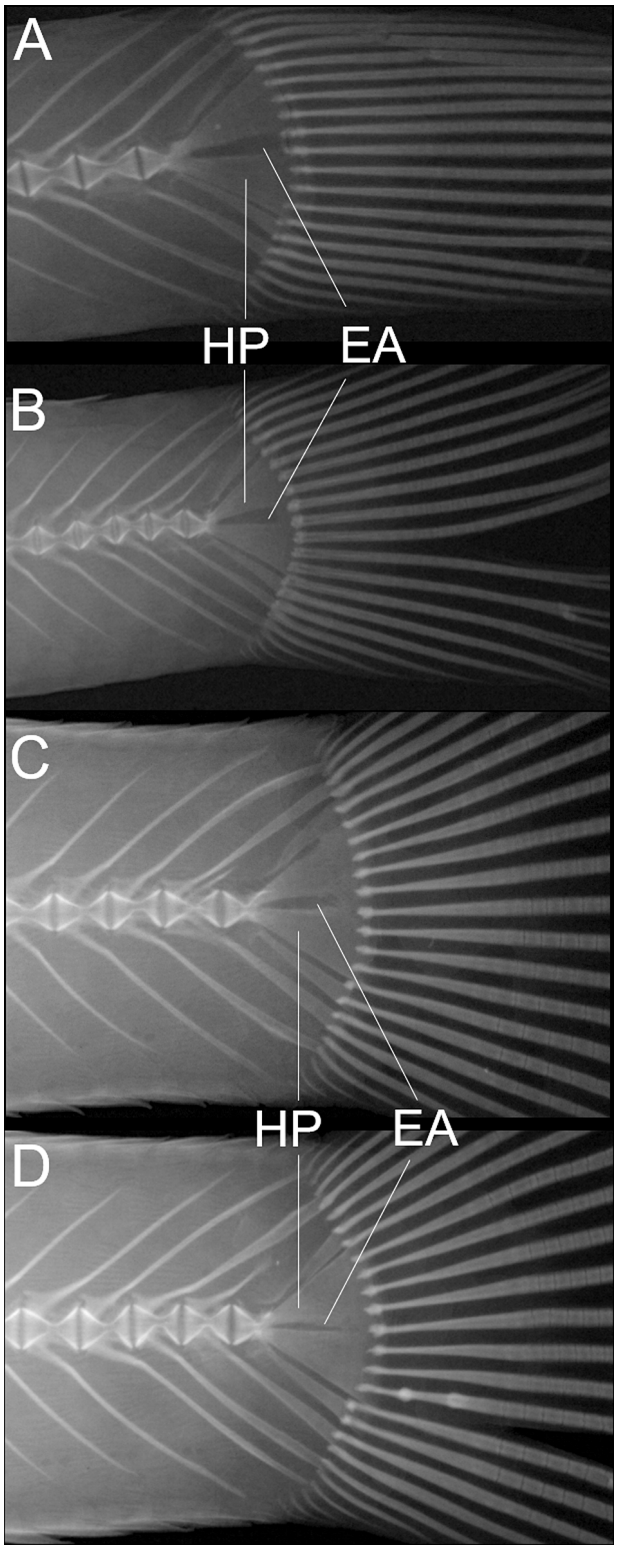

Figure 3. Radiographic images illustrating morphological variation of hypural plates (HP) in *Phalloceros aspilos*, MNRJ43505. **A.** Morphological condition originally described as autapomorphic ("HP almost bipartite with very large aperture", Lucinda, 2008), male, 20.1 mm SL. **B–D.** Variation in the caudal skeleton corresponding to the state "HP partially fused with an elongate aperture" (EA), respectively male and females, 21.2, 31.6, and 29.3 mm SL.

Peixoto, J.R. Amaral, 10 October 2014 (MNRJ43245). Córrego da Toca do Boi, próximo ao portão do condomínio Laranjeiras, município de Parati, no collector data, 3 March 1989 (MNRJ14847, paratypes; MNRJ23609, holotype).

Identification. *Phalloceros enneaktinos* is morphologically very similar to *P. harpagos*. According to Lucinda (2008), *P. enneaktinos* differs from *P. harpagos* by having only 9 dorsal fin rays, but the number of rays is variable in the latter, ranging from 7 to 9. Our survey in the IGBHR of dorsal fin-ray counts among populations of these taxa confirmed the existence of overlapping fin-ray counts (Table 3).

To further evaluate the distinction between these taxa, we compared COI sequences of individuals from the type locality of *P. enneaktinos* with samples of *P. harpagos* from other mainland rivers. Our DNA-sequence comparisons confirmed that the 2 species are genetically distinct (Fig. 4), with a genetic divergence that exceeds accepted levels of intraspecific divergence in fishes (Hebert el al. 2003a, 2003b, Pereira et al. 2013). The Refined Single Linkage Analysis (Ratnasingham and Hebert 2013) performed by the BOLD Systems algorithm assigned the 2 species to distinct Barcode Index Numbers (BIN). Specimens of *P. enneaktinos* were assigned to BIN ACC028, and specimens of *P. harpagos* were assigned to BIN ADM9037. These genetic data also confirmed the unreliable nature of dorsal fin-ray counts as a diagnostic character, because the sample from the Lopes Mendes beach on Ilha Grande island is conspecific with *P. enneaktinos* in spite of these specimens having a modal count of 8 dorsal fin rays. Unfortunately, variation in dorsal fin-ray counts renders the unambiguous identification of samples of *P. enneaktinos* from localities situated far from its type locality very difficult without molecular data.

Table 3. Dorsal fin-ray counts in adults of the *Phalloceros enneaktinos* + *P. harpagos* morphological complex from IGBHR. Locality numbers correspond to those in Table 1. Samples of *P. enneaktinos* associated with DNA sequencing are indicated with an asterisk, and samples of *P. harpagos* associated with DNA sequencing are indicated with 2 asterisks (details in Table 2). Toca do Boi is the type locality of *P. enneaktinos*.

Drainage	Dorsal fin rays			
	7	8	9	10
1-Lopes Mendes*				
males		9	2	
females		18		
6-Caputera**				
males	1	27	2	
females		26	1	
8-Bracuí				
males		14		
females		9		
10-Ambrósio				
males		3		
females	3	8		
11-Mambucaba				
males		15		
females		16		
16-Perequê-Açu				
males	1	5		
females		3		
19-Mamanguá				
males		1	9	
females		4	14	
20-Toca do Boi*				
males		6	16	1
females		5	138	

Distribution. *Phalloceros enneaktinos* was previously known only from the rio Toca do Boi, a small coastal drainage, which flows directly into the Atlantic Ocean outside of Ilha Grande Bay (Lucinda 2008). Here we record the presence of this species in a small stream that flows into the Saco do Mamanguá inlet, which is located inside Ilha Grande Bay, and in a wet area behind Lopes Mendes beach, which is on the southeastern portion of Ilha Grande (Fig. 1).

Phalloceros harpagos Lucinda, 2008

Figure 2D

Materials examined. Brazil: Rio de Janeiro: Rio Caputera ou Areia do Pontal, sob a ponte da rodovia Rio-Santos (BR-101), município de Angra dos Reis, D.F. Belote, 5 February 2003 (MNRJ24250). Rio Caputera ou Areia do Pontal, 2 Km a montante da ponte sobre o mesmo rio (rodovia Rio-Santos, BR-101), município de Angra dos Reis, D.F. Belote, 5 February 2003 (MNRJ42914). Rio Caputera, 9.2 Km (em linha reta) a montante da ponte da rodovia Rio-Santos (BR-101), município de Angra dos Reis (22°56′29″ S, 044°18′41.1″ W), P.A. Buckup, D.F. Moraes Jr, I.C.A. Souto-Santos, M.M. Gonzalez, D.R.S. Peixoto, J.R. Amaral, 12

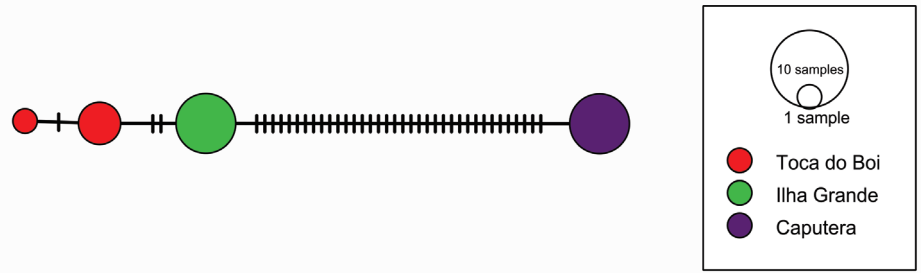

Figure 4. Haplotype network of cytochrome oxidase I (COI) in *Phalloceros enneaktinos* (red and green circles) and *P. harpagos* (purple circles).

October 2014 (MNRJ43508). Rio Ambrósio, a jusante da ponte da BR-101, em Frade (=Cunhambebe), município de Angra dos Reis (22°57′28″S, 044°26′13″ W), P.A. Buckup, D.F. Moraes Jr, V. de Brito, L. Villa-Verde, G. Beltrão, 3 July 2015 (MNRJ43890). ***Phalloceros* cf. *harpagos*.** Brazil: Rio de Janeiro: Segundo afluente do rio Bracuí, município de Angra dos Reis, H. São-Thiago, D.A. Halboth, J.H.C. Gomes, 1 July 1988 (MNRJ47042). Rio dos Sertões, entrando pelo sítio e restaurante Engenho D'Ouro, Penha, município de Parati (23°13′26″S, 044°45′35″ W), R. Souza-Lima, et al., 25 August 2015 (MNRJ50623). Médio rio dos Sertões, entrando pela trilha do rio da Estiva Preta, entrada a pé pelo sítio Bela Vista no começo do trecho de terra da estrada Paraty-Cunha, aproximadamente Km 13, município de Parati (23°12′32″ S, 044°49′50″ W), R. Souza-Lima, et al., 7 July 2016 (MNRJ50628). Brazil: São Paulo: Rio Mambucaba, junto a ponte do Gavião, São José do Barreiro (22°50′13″ S, 044°34′28″ W), P.A. Buckup, R. Arruda, 6 June 2015 (MNRJ43438).

Identification. As discussed above, *P.* harpagos is genetically distinct from *P. enneaktinos*, but there is overlap in the number of dorsal fin rays present in both species, which was the sole character listed by Lucinda (2008) to distinguish these 2 species.

Distribution. This species is recorded for the first time in the IGBHR, where it was collected in the rio Bracuí and rio Caputera, in the eastern portion of Ilha Grande Bay.

***Phalloceros tupinamba* Lucinda, 2008**
Figure 2E

Materials examined. Brazil: Rio de Janeiro: Fazenda Japuíba, município de Angra dos Reis, L.Travassos, H. Travassos, E. Travassos, 28 April 1945 (MNRJ4226). Fazenda Japuíba, município de Angra dos Reis, P. Miranda-Ribeiro, G.S. Myers, H. Travassos, August 1942 (MNRJ8518, MNRJ8548).

Identification. *Phalloceros tupinamba* is very similar to *P. leptokeras*, another species with female urogenital papilla oriented to the left side. According to Lucinda (2008), *P. tupinamba* has a narrow vertically elongate dark lateral spot covering 1 scale, while the latter has a densely pigmented rectangular spot covering 2 or 3 scales. The spot in all 63 examined sexually mature males from the rio Japuíba covers 1 or 1½ scales, but never 2.

In specimens from the rio Japuíba the lateral spot is positioned between the 14th to 17th scale of the longitudinal series. The position of the spot in the Japuíba population is therefore intermediate between that of the type series of *P. tupinamba* (16th–19th scale) and *P. leptokeras* (14th–16th), as recorded by Lucinda (2008).

Distribution. *Phalloceros tupinamba* had previously been recorded in coastal streams located both to the east and west of the IGBHR and now is recorded for the first time in the IGBHR, where it has been collected in the Japuíba drainage (Fig. 1). The new record is based on 3 samples collected in 1942 and 1945. Recent efforts to collect specimens of *P. tupinamba* were unsuccessful due to increased habitat loss associated with the growth of urban areas and presence of a water-treatment facility, which, during dry periods, diverts the entire water flow of the rio Japuíba for human consumption.

Identification key for *Phalloceros* from Ilha Grande Bay Hydrographic Region.

In the following taxonomic key, the urogenital papilla refers to a fleshy projection located between the anus and the origin of the anal fin of females and the gonopodial appendix refers to a pair of projections at the tip of the modified anal-fin rays of adult males in *Phalloceros*. We follow Lucinda (2008) in the usage of the term "hook" to refer to an anterior skeletal process in the gonopodial appendices, even though this process usually is not hook-shaped. *Phalloceros* enneaktinos cannot be distinguished from *P. harpagos* based on morphological characters, even though the 2 species are genetically distinct. Populations of *Phalloceros* from the Joatinga peninsula have predominantly 9 dorsal fin rays and can be confidently identified as *P. enneaktinos*, but, elsewhere, adequate identification of *P. enneaktinos* requires genetic data. Readers with access to COI gene sequences can compare these sequences with our reference samples (Table 3) using the genetic identification algorithms available on GenBank and BoldSystems.

1a Urogenital papilla of females turned to right. In males, hook present only in left gonopodial appendix (Fig. 2A) ..... ***P. anisophallos***

1b Urogenital papilla of females turned to left or straight, pointing distally. In males, a hook present in both sides of gonopodial appendix **2**

2a Urogenital papilla of females straight pointing distally. In males, hook positioned in distal portion of gonopodial appendix (Fig. 2C, D) .... ***P. enneaktinos* + *P. harpagos***

2b Urogenital papilla of females turned to left. In males, hook positioned in medial portion of gonopodial appendix (Fig. 2B, E) **3**

3a Conspicuous dark spot on side of body .... ***P. tupinamba***

3b Side of body without a spot ..... ***P. aspilos***

Discussion

Prior to the current study only 3 species of *Phalloceros* fishes (*P. aspilos*, *P. enneaktinos*, and *P. anisophallos,*) were recorded from streams of the IGBHR (Lucinda 2008). Here, we record 2 additional species (*P. harpagos* and *P. tupinamba*). The number of river drainages with localities where species of *Phalloceros* have been

recorded increased from 6 to 20. These species represent morphologically distinct lineages and correspond to 3 main types of reproductive anatomy that occur in *Phalloceros*. In females the urogenital papilla situated between the anus and the anal fin can be (1) turned to the left, (2) straight pointing distally, or (3) turned to the right. In males, the distinction among these 3 groups is less pronounced but most species with right-sided female papilla have males with a hook solely present on the left gonopodial appendix. Presumably, the genital morphology serves as a reproductive barrier among sympatric species of *Phalloceros*. This hypothesis is corroborated by the sympatry of species with right-sided urogenital papilla (*P. anisophallos*) with both left-sided and straight papillae in the IGBHR. Conversely, species with left-sided papillae (*P. aspilos*, and *P. tupinamba*) are allopatric, as it is also the case with species bearing straight papillae (*P. enneaktinos*, and *P. harpagos*). *Phalloceros anisophallos* is the only representative of the right-sided papilla clade in the IGBHR. This species has a wide distribution in the area, where it has been found in 85% of the sampled drainages.

Among the species of *Phalloceros* present in the IGBHR, *Phalloceros harpagos* stands out as an unusually widespread and morphologically variable taxon (Lucinda 2008). Specimens from numerous coastal streams from southeastern Brazil as well as from the Paraná and Paraguay river basins have been assigned to this taxon, despite the existence of considerable morphological variation among localities (Lucinda 2008). It is likely that *P. harpagos* sensu Lucinda (2008) represents a species complex, and the morphological limits of the species is regarded as provisional until a more comprehensive study of the morphological variation becomes available. The specimens of the IGBHR assigned to *P. harpagos*, however, are morphologically very similar to specimens from the type locality of *P. harpagos*. Additionally, the type locality itself is located in the rio Paraíba do Sul drainage, which is adjacent to the IGBHR. Thus, it is quite plausible that the IGBHR populations are indeed conspecific with the type series of *P. harpagos*.

Two species of *Phalloceros* (*P. anisophallos*, and *P. enneaktinos*) are present on Ilha Grande, despite the fact that the island is isolated from the mainland by at least 4 km of sea, and the insular freshwater drainages with *Phalloceros* populations are located in the oceanic side of the island about 15 km from the mainland and isolated from the mainland side by a mountain range that crosses the island. The presence of these species on the island is consistent with a hypothesis of former interconnection of coastal rivers during periods of low sea level caused by global Pleistocene glaciation events (Weitzman et al. 1988, Buckup 2011). According to a model of coastal paleodrainages for southeastern Brazil (Thomaz et al. 2015, Thomaz and Knowles 2018), the freshwater drainages from Ilha Grande Bay, including those of the main island (Ilha Grande) were interconnected by a single paleodrainage during the Last Glacial Maximum (26,000–19,000 years before present). Such interconnection would allow for the presence of conspecific populations of *Phalloceros* in currently isolated drainages of the Ilha Grande Bay.

The poor performance of morphological characters originally used to distinguish *P. enneaktinos* from *P. harpagos* highlights the need for use of DNA sequence data for species identification outside of type localities. Our DNA-sequence comparisons confirmed that the 2 species are genetically distinct, with a genetic divergence that exceeds accepted levels of intraspecific divergence in fishes (Hebert el al. 2003a, 2003b, Pereira et al. 2013). The cryptic nature of these species also highlights the need to further investigate the genetic structure of *P. harpagos*. *Phalloceros harpagos* is a widespread taxon with great morphological variation (Lucinda 2008). The polyphyletic nature of *P. harpagos* has recently been suggested in a genomic study where specimens identified as *P. hapagos* have been assigned to 4 species and at least 3 independent evolutionary lineages (Thomaz et al. 2019).

The presence of *Phalloceros enneaktinos* in a tributary of the Mamanguá inlet of Ilha Grande Bay and on Ilha Grande is reported here for the first time. This species was previously known from a single stream (rio Toca do Boi) located on the Ponta da Joatinga peninsula at the western limit of Ilha Grande Bay. This species is restricted to the oceanic slopes of Ilha Grande and the Joatinga peninsula and has not been found in the inner drainages of the IGBHR. Close examination of the topography in the area reveals that a major mountain ridge (mostly 400 m above sea level) separates the Mamanguá drowned river valley and the Toca do Boi river valley from the mainland at the base of the Joatinga peninsula. The headwaters of the 2 small streams, on the other hand, are separated by fairly low terrain, around 100 m high. The mountain ridges in the base of the Joatinga peninsula are roughly aligned with the main mountain ridges found on Ilha Grande. It is thus likely that the high ridge system extending from the base of the Joatinga peninsula to Ilha Grande may have acted as a barrier to the dispersal of *P. enneaktinos* even during the Last Glacial Maximum, when most of the Ilha Grande Bay paleodrainage was exposed above sea level.

Phalloceros tupinamba was originally described based on specimens disjunctly distributed in the rio Macacu and rio Itamambuca (Lucinda 2008). These drainages are located to the east and west of the IGBHR. Our new record of *P. tupinamba* in the IGBHR reduces the distributional gap between those drainages. However, the presence of *P. tupinamba* in close proximity to the type locality of the allopatric *P. aspilos* highlights the morphological similarity among members of the clade of species with left-sided female urogenital papillae, and underscores the need to reevaluate the limits among these species. *Phalloceros aspilos* was previously diagnosed by the configuration of the caudal skeleton, but we found considerable variation in that character in

specimens from the Parati Mirim river drainage. We provisionally regard the 2 taxa as distinct species based on the absence of a dark spot on the flank of the body of *P. aspilos*. The variability of the position of the lateral spot of *P. tupinamba* in the Japuíba river drainage also highlights the need to further investigate the limits among these species because of the partial overlap between the position of the spot in type series of *P. tupinamba* (16th–19th scale) and *P. leptokeras* (14th–16th) limits the use of this character as a diagnostic characteristic to distinguish these species, as suggested in the original description (Lucinda 2008).

Our survey of *Phalloceros* species inhabiting the rivers of the IGBHR illustrates the lack of taxonomic knowledge about the Neotropical freshwater ichthyofauna even in well-studied coastal streams of southeastern Brazil. Our survey of this relatively small region has nearly doubled the number of *Phalloceros* species known to inhabit the area since the publication of Lucinda's (2008) revisionary study. It also demonstrates the need for additional geographic sampling and genetic studies to better understand problems of species delineation that become evident only when taxonomic studies are not restricted to single locations associated with type series.

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Author contributions

ICASS and GLSV identified the specimens. ICASS photographed the material and prepared the map. ICASS and PAB conducted field work and wrote the text. PAB performed the analysis of molecular data. GAF and WBJ conducted molecular laboratory work.

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