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Dismantling the *Phalloceros harpagos* species complex (Teleostei: Poeciliidae): description of a new species from Brazilian highlands based on morphological and molecular evidence

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A new species of *Phalloceros* is described from the upper rio Paraná, the upper rio Paraíba do Sul, and headwaters of coastal rivers in southeastern Brazil. This species is distinguished from congeners by characters of both male and female genitalia, dorsal fin-ray counts, dorsal-profile shape, and pigmentation and position of the vertically elongated dark spot on the flanks. The species has been misidentified in the literature as *P. harpagos*. Our DNA sequence analysis of specimens from the type-locality has clarified that *P. harpagos* is restricted to drainages on the right bank of the middle Paraíba do Sul and to coastal drainages in the metropolitan region of Rio de Janeiro. The new species differs from *P. harpagos* by a flattened, hood-like projection of skin located between the anus and the urogenital papilla in most females, and by a rounded lamellar flap on the distal margin of both gonopodial appendices in small mature males (< 21.2 mm SL). Mitochondrial CO1 gene analysis revealed a 6% genetic divergence between the new species and *P. harpagos*, providing strong support for the distinctiveness of this new species.

Keywords: DNA barcode, One-spot livebearer, Serra da Mantiqueira, Serra do Mar, Upper Paraná.

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Uma nova espécie de *Phalloceros* é descrita do alto rio Paraná, alto rio Paraíba do Sul e cabeceiras de rios costeiros no sudeste do Brasil. A espécie distingue-se das congêneres por caracteres da genitália masculina e feminina, contagens de raios da nadadeira dorsal, formato do perfil dorsal e pigmentação e posição da mancha escura verticalmente alongada nos flancos. A nova espécie foi identificada erroneamente na literatura como *P. harpagos*. Nossa análise de sequências de DNA de espécimes da localidade tipo esclareceu que *P. harpagos* é restrita a drenagens na margem direita do médio Paraíba do Sul e bacias costeiras na região metropolitana do Rio de Janeiro. A nova espécie difere de *P. harpagos* pela presença de uma projeção laminar de pele em forma de capuz localizada entre o ânus e a papila urogenital da maioria das fêmeas, e uma aba lamelar arredondada na margem distal de ambos os apêndices gonopodiais em machos maduros pequenos (< 21,2 mm CP). A análise do gene mitocondrial CO1 revelou uma divergência genética de 6% entre a nova espécie e *P. harpagos*, fornecendo forte suporte para a distinção da nova espécie.

Palavras-chave: Alto Paraná, Código de barra de DNA, Guaru, Serra da Mantiqueira, Serra do Mar.

INTRODUCTION

The monophyletic genus *Phalloceros* Eigenmann, 1907 comprises 21 valid species of livebearer fishes distributed in freshwater habitats of southeastern South America. These species occur in coastal drainages from the southern portion of the Brazilian State of Bahia to Uruguay, extending westward to the La Plata drainage in Argentina and Paraguay, and reaching the Tocantins-Araguaia basin (Lucinda, Reis, 2005; Lucinda, 2008; Souto-Santos *et al*., 2023a,b).

Similar to other poeciliid livebearers, species of *Phalloceros* exhibit pronounced sexual dimorphism. Mature males possess elongated third, fourth, and fifth anal-fin rays that form a copulatory organ called gonopodium, whereas mature females have a prominent genital papilla located posterior to the anus. *Phalloceros* can be diagnosed by the presence of a distal pair of appendices at the tip of the third ray of the gonopodium (character state 93–1 in Lucinda, Reis, 2005; Lucinda, 2008), and the partial opening of the preopercular sensory canal, which forms a groove between pores 8 and 10, sometimes between pores 8 and 11, and occasionally between pores V and U; pore 11 may be elongate when present as a separate opening (Lucinda, 2008; character state 8–5 in Lucinda, Reis, 2005; pore nomenclature following Gosline, 1949).

Phalloceros harpagos Lucinda, 2008 was described based on specimens from the upper rio La Plata system and coastal basins between the rio Itabapoana in the Brazilian State of Espírito Santo and the rio Araranguá in the southern State of Santa Catarina. This extensive geographic range is much larger than that of the other species in the genus. The hypothesis that *P. harpagos* constitutes a species complex was considered by Lucinda (2008) in the original description, but it could not be corroborated by the morphological evidence available at the time. However, the polyphyletic nature of the

"*P. harpagos*" morphotype has been demonstrated in two recent studies, where specimens identified as *P. harpagos* have been assigned to multiple independent evolutionary lineages (Thomaz *et al*., 2019; Oliveira *et al*., 2024).

As part of a long-term study on *Phalloceros* diversity, we sequenced, for the first time, a segment of the CO1 (cytochrome c oxidase subunit 1) gene from topotype specimens of *P. harpagos*. This enabled us to genetically characterize this species and distinguish it from its congeners through the integration of molecular and morphological data. In this study, we contribute to resolving the *P. harpagos* species complex by describing a formerly cryptic species from the upper rio Paraná and adjacent river basins that was included in the original concept of *P. harpagos*.

MATERIAL AND METHODS

Specimen preparation and measurements. Counts of bony elements were based on cleared and stained or radiographed specimens (Fig. **S1**). Clearing and staining followed the method of Taylor, Van Dyke (1985), omitting cartilage staining steps. Measurements and counts of bilateral structures were obtained from the left side of the body, except for tooth counts, which included teeth from both sides, following Lucinda (2005). Measurements followed Lucinda (2005, 2008), with distances taken point-to-point to the nearest 0.1 mm using digital calipers under a stereomicroscope. Body measurements are expressed as percents of standard length (SL), except for subunits of the head, which are expressed as percents of head length (HL).

Specimen selection and description. In the species description, numbers in parentheses represent the total number of individuals with the associated counts, and asterisks indicate counts of the holotype. Type-specimens were selected from the rio Grande drainage (a tributary of the upper rio Paraná basin), and specimens examined from other basins were listed as non-type specimens because they were not formally included in the table of morphometric measurements. Morphometric and meristic data were obtained only from adult individuals. Adult males are characterized by a fully developed pair of appendices at the tip of the gonopodium, with exposed hooks (skeletal processes in the anterior portion of the appendices; Lucinda, 2008). Adult females are identified by a urogenital papilla located between the anus and the anal fin. Gonopodium nomenclature follows Rosen, Gordon (1953) as modified by Lucinda, Reis (2005).

Species concept and diagnosis. We adopted the phylogenetic species concept *sensu* Mishler, Theriot (2000): species are the smallest monophyletic groups of organisms worthy of a formal recognition within a classification scheme. Our morphological diagnosis of the newly proposed species is presented as a hierarchy of differential characters separating the new species from groups of congeneric species.

Examined material. Data in the list of examined material are presented in the following sequence: collection acronym (*sensu* Sabaj, 2020), catalog number, number of examined specimens in ethanol, number of cleared and stained specimens (separated from number in ethanol by a slash), SL range in the lot, type status, country, state, municipality, collection locality, geographic coordinates, date, and collector(s). Brazilian Portuguese nouns are used to describe the various types of natural water courses. Toponyms maintain the original Portuguese spelling.

Molecular analysis. Genomic DNA was extracted from muscle tissue preserved in anhydrous ethanol using either the Qiagen Blood and Tissue kit following the manufacturer's instructions, or the salting out method by Miller *et al*. (1988). DNA quality was verified through standard agarose gel electrophoresis, and DNA concentration was measured using a NanoDrop ND-2000 spectrophotometer. Partial sequences of the cytochrome c oxidase subunit I (CO1) were amplified via polymerase chain reaction (PCR) using FishF1 (5′ CA ACC AAC CAC AAA GAC ATT GGC AC 3′) and FishR1 (5′ TAG ACT TCT GGG TGG CCA AAG AAT CA 3′) primers developed by Ward *et al*. (2005). PCR product quality was verified using 2% agarose gel electrophoresis, and the products were purified using ExoSAP-IT (Bell, 2008). Each PCR product was sequenced bidirectionally on an ABI3730xl (Applied Biosystems) automated sequencer at the Fundação Oswaldo Cruz (FIOCRUZ).

Sequences were aligned to a reference sequence using the GENEIOUS v. 6 software (http://www.geneious.com) and manually edited to fine-tune base calls and ensure codon alignment. The sequences contain 655 nucleotides with no missing data. Genetic distance calculations were performed with MEGA X under Kimura 2-parameter model (Kumar *et al*., 2018). A Maximum Likelihood analysis was applied using W-IQ-TREE (Trifinopoulos *et al*., 2016) with default parameters and codon partitioning. The tree was rooted in *Poecilia vivipara* Bloch & Schneider, 1801 (GenBank accession: MW807384), and includes 101 sequences of the new species and morphologically similar congeners. A haplotype network was inferred for the new species using the TCS method (Templeton *et al*., 1992) on PopART (Leigh, Bryant, 2015).

Details about the tissue samples used for DNA sequencing are listed in Tab. **S2**. Sequences were obtained from 11 samples from the upper rio Paraná basin (Paranaíba, Grande, Tietê, Iguaçu, Iguatemi), and 23 from coastal basins, encompassing its entire range. The resulting DNA sequences are available in the BoldSystems (http://www.boldsystems.org/) and NCBI (https://www.ncbi.nlm.nih.gov/genbank/) public databases.

RESULTS

Phalloceros circummontanus, new species

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(Figs. 1A, 2A; Tab. 1)

Phalloceros harpagos (Tietê). —Thomaz *et al*., 2019:270, fig. 3 (hypothesis of phylogenetic relationships among congeners).

Phalloceros harpagos (Haplotype 5). —Oliveira *et al*., 2024:5, fig. 2 (Haplotype 5 clade in a gene tree with results of four molecular species delimitation analyses).

Holotype. MNRJ 55366, 25.7 mm SL, female, Brazil, Minas Gerais, Caldas, small stream on the left bank of the rio Pardo (upper rio Paraná basin: rio Grande drainage), on the road from Caldas to Fim dos Campos, 400 m east of the road to São Pedro de Caldas, 21°54'45"S 46°19'30"W, 22 Nov 2003, P. A. Buckup & R. Souza-Lima.

Paratypes. All from Brazil, State of Minas Gerais: rio Grande drainage (tributary of upper rio Paraná basin). **Rio das Mortes drainage:** MNRJ 31883, 113, 12.3–36.7 mm SL, Carandai, rio Carandaí, upstream from Carandaí, Ponte Alta neighborhood, 20°57'12"S 43°46'34"W, 2 Sep 2007, P. A. Buckup, M. R. Britto, U. Jaramilo, L. Villa-

FIGURE 1 | Species comparisons in *Phalloceros*. **A.** *P. circummontanus*: holotype, ♀ 25.7 mm SL, MNRJ 55366; paratype, ♂ 17.5 mm SL, MNRJ 26119. **B.** *P. enneaktinos*, topotypes: ♀ 24.9 mm SL, ♂ 22.9 mm SL, MNRJ 43245. **C.** *P. harpagos*, paratypes: ♀ 31.4 mm SL, ♂ 25.1 mm SL, MNRJ 14745. **D.** *P. titthos*, topotypes: ♀ 24.3 mm SL, ♂ 18.1 mm SL, MNRJ 52359.

TABLE 1 | Morphometric data for *Phalloceros circummontanus* based on holotype, MNRJ 55266, and paratypes, MNRJ 26119 (16), MCP 55062 (3). Distances measured with calipers based on landmarks described in Lucinda (2005). N = number of specimens; H = holotype; SD = standard deviation. Female range includes the holotype.

Landmarks	Distance	Females (N = 11)				Males (N = 9)		
		H	Range	Mean	SD	Range	Mean	SD
1–5	Standard length (mm)	25.7	19.5–26.0	22.6	–	15.4–19.5	17.3	–
Percents of standard length								
1–2	Snout-occipital distance	19.8	16.7–22.2	19.8	1.8	16.9–21.7	19.7	1.5
1–3	Predorsal distance	63.0	59.8–69.7	62.9	2.6	56.0–61.3	57.8	2.1
3–4	Dorsal-fin base length	8.6	8.5–10.5	9.4	0.7	7.1–11.6	10.0	1.3
8–9	Anal-fin base length	10.1	9.2–12.0	10.2	0.8	5.8–8.0	7.0	0.8
3–8	Body depth	25.7	22.2–25.7	24.0	1.1	30.3–35.5	32.2	1.5
1–10	Pre-pelvic length	44.7	40.6–46.9	44.7	1.6	27.8–31.8	29.7	1.2
1–8	Preanal length	58.0	51.9–58.0	55.5	2.0	35.2–40.3	39.0	1.6
5–8	Postanal length	44.4	41.5–46.6	44.4	1.3	61.9–65.7	63.6	1.3
6–7	Caudal peduncle depth	14.0	8.5–15.0	13.4	1.8	13.7–15.1	14.4	0.4
1–13	Head length	23.7	21.8–26.2	24.0	1.4	21.7–24.7	23.0	1.0
Percents of head length								
1–11	Snout length	19.7	12.2–22.2	17.8	3.1	15.4–19.5	17.3	1.1
11–12	Orbital diameter	34.4	32.8–40.0	36.7	2.2	2.9–3.8	3.4	0.3
12–13	Postorbital length	41.0	41.0–50.0	44.4	2.6	9.4–11.0	10.0	0.5

Verde & J. Maldonado. MNRJ 54586, 65, 10.3–25.3 mm SL, Santa Cruz de Minas, tributary of the Rio das Mortes, downstream of Bom Despacho waterfall, near Min. Gabriel Passos Avenue, 21°7’15”S 44°12’16”W, 22 May 2023, I. C. A. Souto-Santos, B. C. A. Souto-Santos, D. C. A. Souto-Santos & M. V. Santos. **Rio Sapucaí drainage:** MNRJ 54494, 5, 12.9–41.2 mm SL, Itamonte, rio da Colina, upstream of Itamonte, rio Verde drainage, 22°19’24”S 44°50’20”W, 20 May 2023, I. C. A. Souto-Santos, B. C. A. Souto-Santos, D. C. A. Souto-Santos & M. V. Santos. MNRJ 54589, 9, 18.5–28.5 mm SL, Carmo do Rio Claro, Pedra Molhada village, 21°00’18”S 46°14’49”W, 30 Oct 2021, A. Katz & V. M. Azevedo-Santos. MNRJ 23975, 172, 13.0–42.6 mm SL, São Bento do Sapucaí, córrego Monjolinho, at bridge about 1.5 km east of road SP-042, 22°42’50”S 45°42’15”W, 31 May 2002, P. A. Buckup, L. Ingenito, A. T. Aranda, C. Chamon & F. P. Silva. **Rio Pardo drainage:** MNRJ 26119, 52/4, 8.9–25.3 mm SL, MCP 55062, 20, 9.3–26.0 mm SL, collected with the holotype.

Non-type specimens. All from Brazil, grouped according to hydrographic drainage from north to south. **Upper rio Paraná basin, Paranaíba:** MNRJ 51292, 6, 16.4–30.9 mm SL, Distrito Federal, Brasília, ribeirão do Torto (tributary of rio São Bartolomeu), Torto, Vila dos Operários, 15°41’59”S 47°54’28”W, 13 Sep 2018, F. H. Soares. MNRJ 54227, 1, 18.6 mm SL, Distrito Federal, Brasília, ribeirão da Gama (drainage of the Paranoá, São Bartolomeu), Brasília airforce base, 15°52’6”S 47°53’28”W, 19 Dec 2022, C. H. Luz. **Tietê:** LBP 14607, 2, 22.2–26.5 mm SL, São Paulo, Botucatu, rio Araquá, 22°44’50”S 48°28’30”W, 25 Nov 2011, F. Roxo, M. Mehana & G. S. C. Silva. ZUEC-PIS 6159, 32, 10.0–22.1 mm SL, São Paulo, Itirapina Ecological Station, approx. 22º14' S 47º53'W, 10 Jan 2002, G. B. MNRJ 54859, 2, 21.4–28.3 mm SL, São Paulo, Valinhos, small stream tributary of rio Atibaia, Dom Pedro I highway bridge, 22°55'49"S 46°56'22"W, 11 Sep 2023, P. A. Buckup, E. Mejia & I. C. A. Souto-Santos. **Iguatemi:** MNRJ 54801, 25, 12.1–33.1 mm SL, Mato Grosso do Sul, Mundo Novo, córrego Santa Maria upstream from BR-163 culverts, right bank tributary of rio Iguatemi, 23°54'56"S 54°17'31"W, 9 Sep 2023, P. A. Buckup, E. Mejia & I. C. A. Souto-Santos. **Lower Iguaçu:** MNRJ 54789, 22, 12.3–26.6 mm SL, Paraná, Santa Terezinha de Itaipu, Ribeirão São João and adjacent floodplains next to a culvert in secondary road paved with stones, 25°32'4"S 54°25'2"W, 3 Sep 2023, P. A. Buckup, E. Mejia & I. C. A. Souto-Santos. **Rio Paraíba do Sul basin, Pomba:** MNRJ 54588, 28, 11.9–36.8 mm SL, Minas Gerais, Oliveira Fortes, rio Formoso, tributary of rio Pomba, on side road of BR-040, 21°21'6"S 43°35'24"W, 24 May 2023, I. C. A. Souto-Santos, B. C. A. Souto-Santos, D. C. A. Souto-Santos & M. V. Santos. **Paraibuna:** MNRJ 54209, 6, 16.2–25.5 mm SL, Minas Gerais, Santa Rita da Jacutinga, Vargem do Sobrado Waterfall, 22°5'13"S 44°8'19"W, 30 May 2022, I. C. A. Souto-Santos, E. B. Neuhaus & A. Barcia. MNRJ 52959, 4, 17.3–26.3 mm SL, Minas Gerais, Santa Rita da Jacutinga, Vargem do Sobrado Waterfall, 22°5'13"S 44°8'19"W, 1 Dec 2021, M. R. Britto, I. C. A. Souto-Santos & E. B. Neuhaus. MNRJ 52952, 52, 14.1–35.8 mm SL, Minas Gerais, Santa Rita da Jacutinga, Ribeirão Jacutinga, rio Preto drainage, 22°8'5"S 44°5'5"W, 1 Dec 2021, M. R. Britto, I. C. A. Souto-Santos & E. B. Neuhaus. **Pedras:** MNRJ 54489, 30, 9.8–28.0 mm SL, Rio de Janeiro, Itatiaia, rio das Pedras at Três Bacias, close to Koskela restaurant at 4065 Três Cachoeiras Avenue, 22°25'14"S 44°32'15"W, 19 May 2023, I. C. A. Souto-Santos, B. C. A. Souto-Santos, D. C. A. Souto-Santos & M. V. Santos. **Caçapava Velha:** MNRJ 54504, 7, 14.4–30.6 mm SL, São Paulo, Caçapava, creek on corner of SP-062 and Egydia Maria Street, 23°4'1"S 45°39'45"W, 1 July 2022, E. B. Neuhaus & V. C. Gomes. **Barreiro de Baixo:** MNRJ 41749, 1, 18.1 mm SL, Rio de Janeiro, Porto Real, rio Barreiro de Baixo, Resende – Bulhões road, 22°27'57"S 44°22'1"W, 8 Nov 2013, P. A. Buckup & T. S. Parente. **Comando:** MNRJ 24441, 44, 8.6–31.4 mm SL, São Paulo, Jacareí, next to bridge south of Colonião neighborhood, east of Jacareí, 23°17'46"S 45°53'43"W, 16 Feb 2002, P. A. Buckup, F. P. Silva & R. Souza-Lima. AMNH 22673, 11, 15.5–26.1 mm SL, São Paulo, Jacareí, rio Paraíba do Sul, 14 July 1908, J. D. Haseman. **Ilha Grande Bay, Mambucaba:** MNRJ 43438, 50, 10.3–27.8 mm SL, São Paulo, São José do Barreiro, rio Mambucaba next to Gavião bridge, 22°50'13"S 44°34'28"W, 6 Jun 2015, P. A. Buckup & R. Arruda. **Perequê-Açu:** MNRJ 50623, 1, 22.3 mm SL, Rio de Janeiro, Paraty, rio Sertões, entering through Engenho D'Ouro restaurant at Penha, 23°13'26"S 44°45'35"W, 25 Aug 2015, R. Souza-Lima and others. MNRJ 50628, 16, 13.2–34.2 mm SL, Rio de Janeiro, Paraty, middle rio Sertões, trail to rio Estiva Preta from gate of Sítio Bela Vista at beginning of dirt section of Paraty-Cunha road, approximately km 13, 23°12'32"S 44°49'50"W, 7 Jul 2016, R. Souza-Lima and others. **Rio Itapanhaú basin:** MNRJ 41734, 12, 11.9–34.6 mm SL, São Paulo, Mogi das Cruzes, headwaters of rio Grande, Natureza de Taiacupeba District, 23°41'52"S 46°8'23"W, 7 Nov 2011, P. A. Buckup, D. F. Almeida, R. Souza-Lima & R. R. Rodrigues.

Diagnosis. The *Phalloceros circummontanus* is a member of a group of species (also including *P. caudimaculatus* (Hensel, 1868), *P. elachistos* Lucinda, 2008, *P. enneaktinos* Lucinda, 2008, *P. harpagos*, *P. heptaktinos* Lucinda, 2008, *P. leticiae* Lucinda, 2008, *P. maldonadoi* Souto-Santos, Lucinda & Buckup, 2023, *P. mikrommatos* Lucinda, 2008, *P. ocellatus* Lucinda, 2008, and *P. titthos* Lucinda, 2008) with the tip of the female urogenital papilla oriented caudally (*vs*. tip curved to the right in *P. alessandrae* Lucinda, 2008, *P. anisophallos* Lucinda, 2008, *P. buckupi* Lucinda, 2008, *P. lucenorum* Lucinda, 2008, *P. malabarbai* Lucinda, 2008, *P. megapolos* Lucinda, 2008, *P. pellos* Lucinda, 2008, *P. reisi* Lucinda, 2008, *P. spiloura* Lucinda, 2008, and *P. uai* Lucinda, 2008; tip curved to the left in *P. leptokeras* Lucinda, 2008). *Phalloceros circummontanus* is distinguished from *P. caudimaculatus*, *P. heptaktinos*, *P. leticiae*, *P. mikrommatos*, and *P. ocellatus* by the possession of a hook in the gonopodial appendix (*vs*. hook absent in the gonopodial appendix); from *P. titthos*, by the convexity of the dorsal profile between the tip of the snout and the origin of the dorsal fin in both males and females (*vs*. dorsal profile straight); from *P. enneaktinos*, by having a dark pigmented lateral spot located modally in the 16th scale (*vs*. spot absent or difuse, located modally in the 18th scale) (Tab. 2), and the possession of seven or eight dorsal-fin rays, very rarely nine (*vs*. nine, very rarely eight or ten) (Tab. 3); from *P. harpagos* and *P. elachistos*, by having a posterior rounded flap on the gonopodial appendix in small (15.4–21.2 mm SL) specimens (*vs*. flap absent in all size classes of mature males). Additionally, *P. circummontanus* differs from *P. harpagos* by having a flattened hood-like projection of skin located between the anus and the urogenital papilla of most females (*vs*. hood-like projection of skin always absent); and from *P. maldonadoi* by the symmetrical hood or hood absent (*vs*. asymmetrical hood always present).

TABLE 2 | Lateral spot position in *Phalloceros circummontanus* and *P. enneaktinos*. The lateral spot of *P. circummontanus* is more anterior and more pigmented (see Fig. 1). The lateral spot of *P. enneaktinnos* is more posterior, diffuse or absent.

Species	Drainage	Lateral spot position (scales as reference)							
		No spot	14th	15th	16th	17th	18th	19th	20th
P. circummontanus	Grande: Mortes								
	males		1	5	14	17	2		
	females		5	10	60	27			
P. circummontanus	Grande: Sapucaí								
	males				1	2			
	females				1	3	1		
P. circummontanus	Grande: Pardo								
	males				1	5	3		
	females				4	6	1		
P. enneaktinos	Toca do Boi								
	males	7					5	13	
	females	34				6	48	29	1

TABLE 3 | Dorsal fin-ray counts in adults of *Phalloceros circummontanus* and *P. enneaktinos*.

Species	Drainage	Dorsal-fin rays			
		7	8	9	10
P. circummontanus	Upper Paraná: Grande: Mortes				
	males	5	5		
	females	14	54	1	
P. circummontanus	Upper Paraná: Grande: Sapucaí				
	males		2	1	
	females		3		
P. circummontanus	Upper Paraná: Grande: Pardo				
	males		9		
	females		16	2	
P. circummontanus	Mambucaba				
	males		15		
	females		16		
P. circummontanus	Perequê-Açu				
	males	1	5		
	females		3		
P. enneaktinos	Mamanguá				
	males		1	9	
	females		4	14	
P. enneaktinos	Toca do Boi				
	males		6	16	1
	females		5	138	
P. enneaktinos	Lopes Mendes				
	males		9	2	
	females		18		

Description. Morphometric data of holotype and paratypes in Tab. 1. Overall appearance shown in Fig. 1A. Body elongate, fusiform. Greatest body depth at dorsal-fin origin. In lateral view, dorsal profile convex from snout to dorsal-fin origin, posteroventrally slanted at dorsal-fin base, nearly straight at caudal peduncle. Ventral profile of head convex at lower jaw, nearly straight from anguloarticular to isthmus. In females, ventral profile increasingly convex from isthmus to anal-fin origin, slightly posterodorsally slanted at anal-fin base, straight at caudal peduncle. In males, ventral profile straight from isthmus to anal-fin origin, slightly convex at caudal peduncle. Distal margin of dorsal fin gently curved. Distal margin of caudal fin rounded. In adult males, base of anal and pelvic fins displaced to anterior portion of belly. Gonopodium base wider than tip. Distal margin of anal fin in females nearly straight. Pectoral fin rounded, symmetrical. Mouth superior. Dermal papillae present on symphyseal portion of lower lip. Maxilla vertically oriented; posterior tip of maxilla not reaching level of anterior margin of orbit. Nares dorsally positioned; anterior and posterior nasal pores separated. Orbital margin circular. Margin of eye free. Teeth unicuspid. Outer premaxillary teeth 14(1), 15(2) or 18(1). Outer dentary teeth 10(1), 12(1), 18(1) or 19(1). Maxilla and vomer toothless. Fourth ceratobranchial toothless; inner surface of fifth ceratobranchial covered with numerous unicuspid teeth. Branchiostegal rays 5(4). Scales cycloid, 28(3) or 29*(17) in longitudinal series, 7(7) or 8*(13) in transverse series.

Series of scales around caudal peduncle 15(2) or 16*(18). Dorsal-fin rays 7(19), 8*(89) or 9(4). Branched pectoral-fin rays 5(1), 6(12) or 7*(3). Pelvic-fin rays 5(4). Anal-fin rays in females 10(8) or 11*(3). Anal-fin rays in males 9(2). Branched caudal-fin rays 11*(5) or 12(10). Predorsal scales 14(1), 15*(14) or 16(5). Epipleural ribs 12(4). Pleural ribs 13(2) or 14(2). Vertebrae 31(3) or 32*(2).

Genital morphology. Adult females larger than males, with anal-fin base between vertebrae 15 and 19; anal fin fan shaped; abdominal cavity surrounded by all ribs. Adult females with urogenital papilla located between anus and anal fin (Fig. 2). Papilla usually covered anteriorly by an expanded dermal flap (*i.e.*, a "hood", Souto-Santos *et al.*, 2023b); hood absent in some large specimens (Fig. 3). Papilla pointing caudally; base of papilla aligned with anus and anal fin. Hood much thinner than papilla. Hood and papilla fused near their base on both sides.

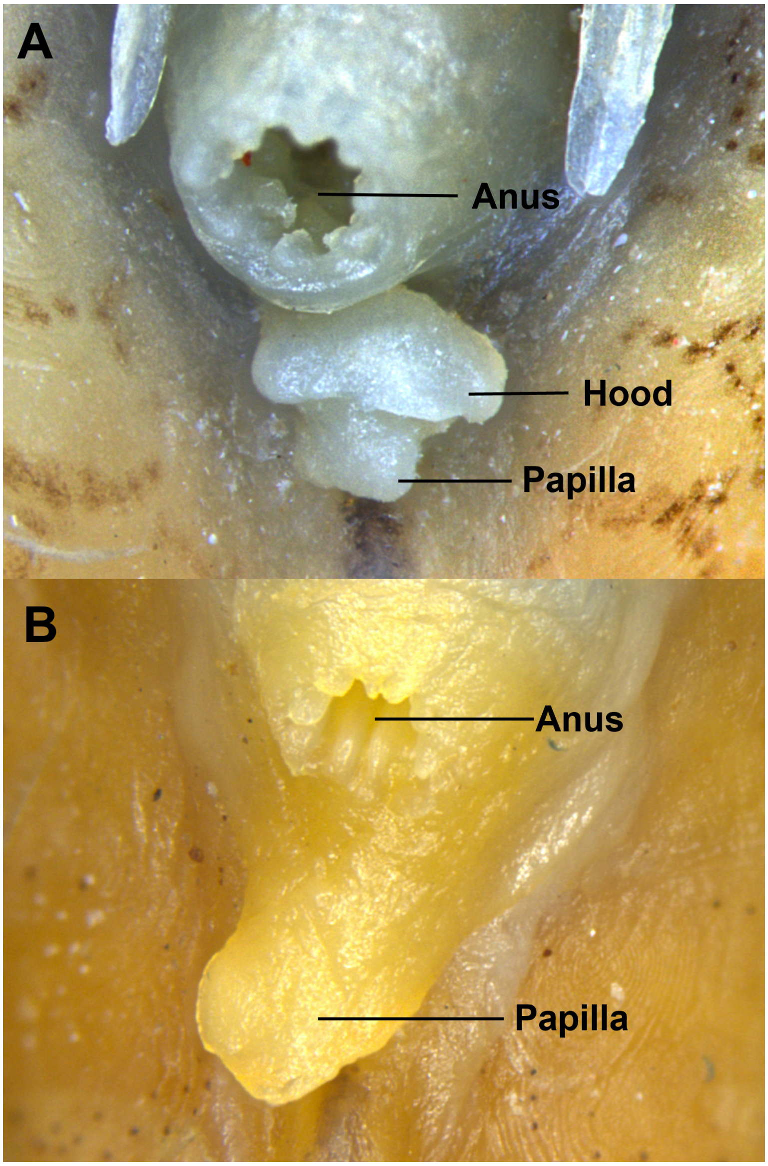

FIGURE 2 | Comparison of female urogenital area. **A.** *Phalloceros circummontanus*, MNRJ 26119, 22.4 mm SL, paratype. **B.** *P. harpagos*, MNRJ 14745, 33.5 mm SL, paratype.

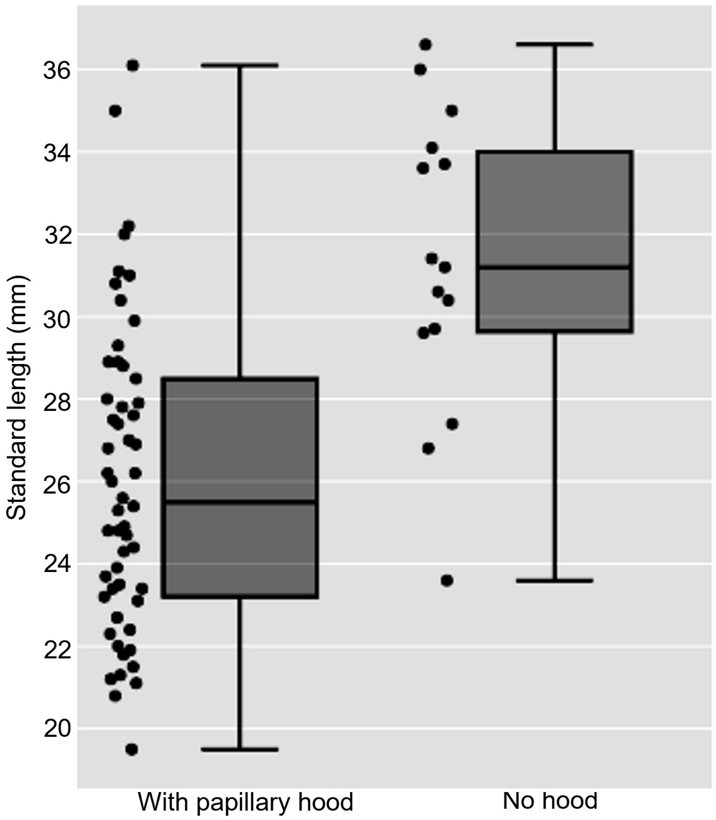

FIGURE 3 | Size comparison between females of *Phalloceros circummontanus* with genital hood (left, N = 54, median = 25.5), and without genital hood (right, N = 15, median = 31.2), demonstrating that the hood is present in all sizes, but occasionally absent in large specimens. Note that the intervals between the lower and upper quartiles do not overlap. See Tab. **S3** for individual measurements and voucher codes.

Adult males with anal-fin base between vertebrae 9 and 13; anal fin spear-shaped; abdominal cavity between posterior ribs occupied by hypertrophied haemal spines (= gonapophyses), modified anal-fin pterygiophores and associated muscles; anal-fin rays 3 to 5 greatly elongate, modified into gonopodium. Gonopodium of adult males with conspicuous ornamentation at tip (Fig. 4). Serrae on posterior branch of the fourth ray-R4p 9(4), 10(3). Pair of appendices coalesced at base, on tip of third ray-R3. Each appendix with distal curvature, bearing hook at point of inflection. Distal half of appendix narrower than proximal half; narrowest region located between hook insertion and tip of appendix. Each appendix usually with rounded, distal flap of skin in small males (< 22 mm SL); flap absent in larger males (> 22 mm SL) (Fig. 5). Juvenile males without appendices or with skin covering hooks. Gonopodial suspensory with three large gonapophyses projected anteriorly, from ventral surface of 14th to 16th vertebrae (Fig. **S1**). Gonapophysis-bearing vertebrae also bearing posteriorly-directed parapophyses with ribs.

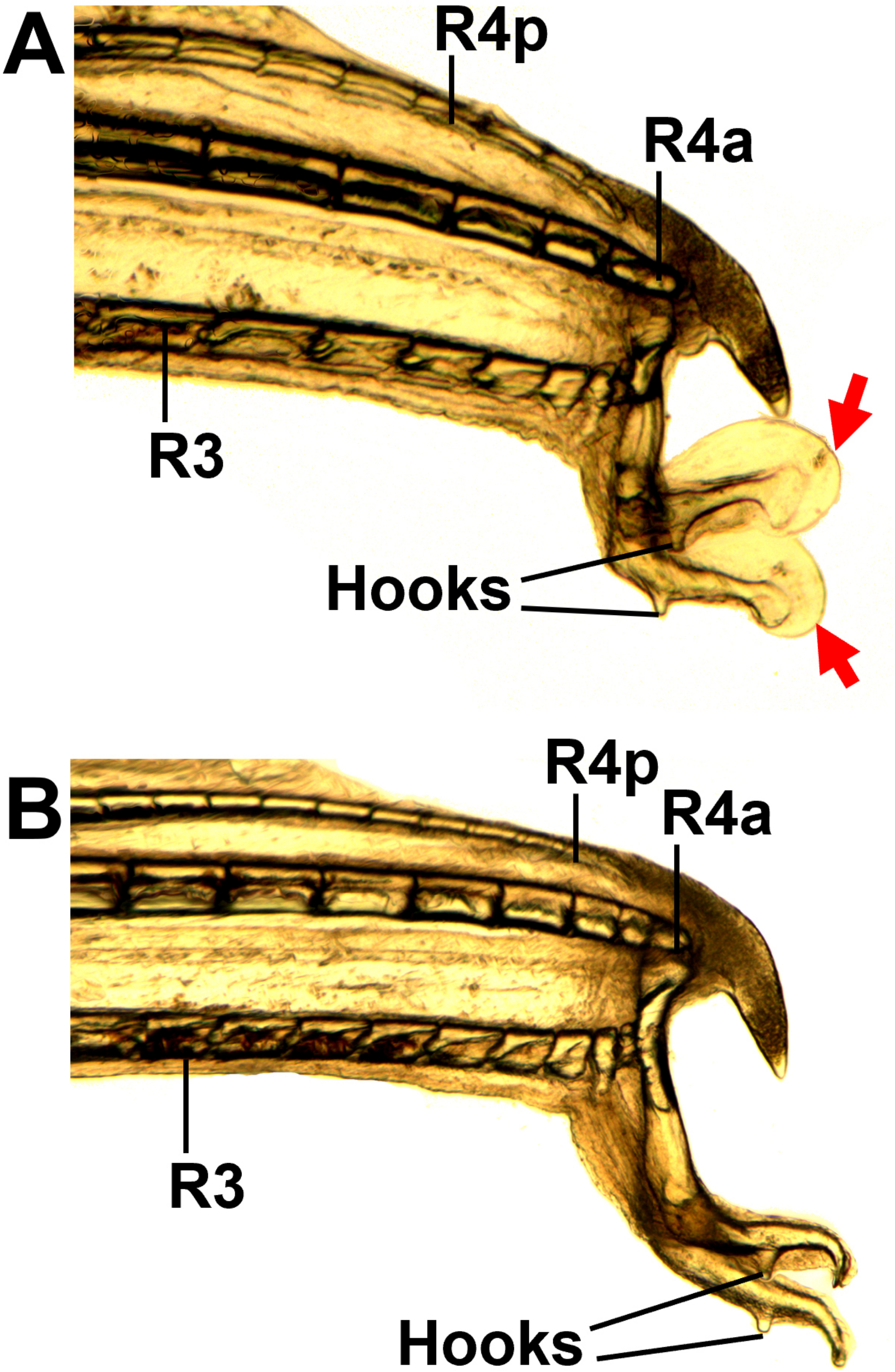

FIGURE 4 | Distal region of gonopodium in *Phalloceros circummontanus*, MNRJ 31883, paratypes. **A.** 18.3 mm SL. **B.** 22.2 mm SL. Red arrows indicate dermal flaps around gonopodial appendices. R3 = Third ray, R4a = Fourth ray, anterior branch, R4p = Fourth ray, posterior branch.

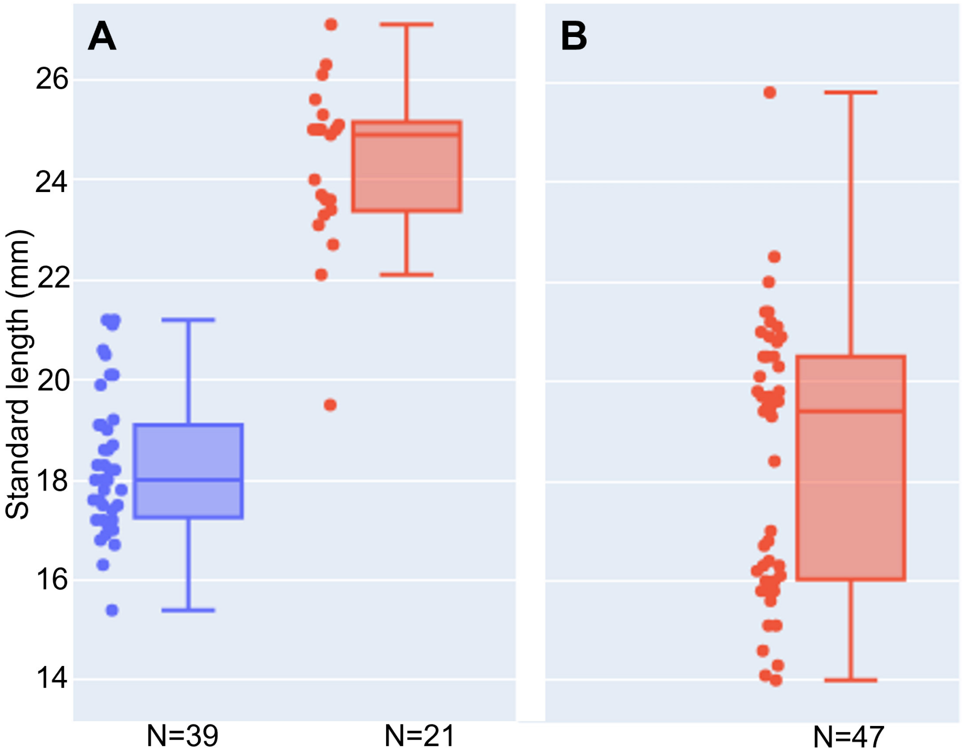

FIGURE 5 | Size comparison of male *Phalloceros*. **A.** *P. circummontanus* with (blue boxplot, median = 18.0) and without (red boxplot, median = 24.9) dermal flaps around the tips of gonopodial appendices. The intervals of the boxplots do not overlap. **B.** Size variation of *P. harpagos* males without dermal flaps (median = 19.4). See Tab. **S4** for individual measurements and voucher codes. Note that dermal flaps are present in early ontogenetic stages of *P. circummontanus*, but absent in *P. harpagos* even at small individual sizes.

Color in ethanol after field fixation in formalin. Background color burlywood, darker on dorsal region. Eye black with greenish grey pupil. Posterior border of scales and underlying skin with numerous brown chromatophores, most concentrated at short distance from scale posterior border, conferring reticulate pattern to sides of body, mainly on dorsal region. Brown or black chromatophores scattered throughout body skin, more concentrated on dorsum, snout, opercle, and ventral surface of mandible. Conspicuous vertically elongate dark spot on flank, located posteriorly to dorsal-fin origin, on 14th(6), 15th(15), 16th*(81), 17th(60) or 18th(7) scale of longitudinal series, covering one(3), one-and-a-half*(12) or two(5) scales on horizontal axis, one(2), one-and-a-half(2), two*(14), two-and-a-half(1) or three(1) scales on vertical axis. Light vertical bars on flank in some specimens. Dorsal-fin membrane hyaline bearing dark band of chromatophores on distal border of fin and another near fin base. Pectoral, pelvic, and caudal fins hyaline. In males, chromatophores along R3 of gonopodium forming dark line or diffuse pigmentation. First three anal-fin rays of females often with chromatophores forming patch of dark pigmentation.

Geographical distribution. *Phalloceros circummontanus* is widely distributed in the rio Paraná basin, encompassing the Brazillian states of Goiás, Minas Gerais, São Paulo, Paraná, and Mato Grosso do Sul. The species is also known from the upper rio Paraíba do Sul basin, and coastal basins from the rio Mambucaba in the State of Rio de Janeiro to rio Itapanhaú in the State of São Paulo (Fig. 6).

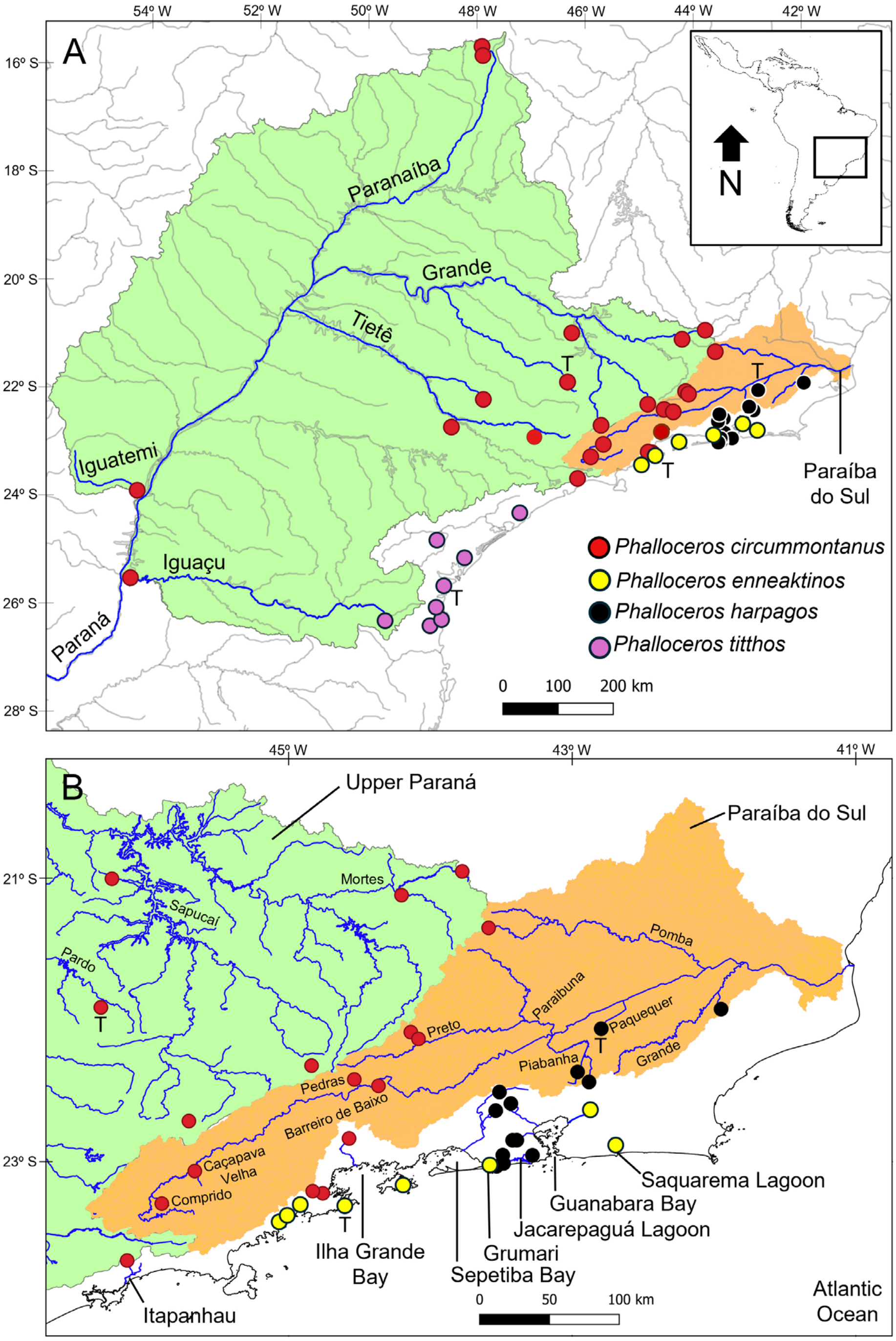

FIGURE 6 | Known geographic distribution of *Phalloceros circummontanus*, including updated distribution of *P. enneaktinos*, *P. harpagos*, and *P. titthos*. **A.** Global view of the study area. **B.** Close-up of the watershed between the upper Paraná and Paraíba do Sul. See Tab. **S2** for voucher catalog codes and field data details of the previously described species (records confirmed by DNA sequencing). T = Type-locality.

Ecological notes. *Phalloceros circummontanus* occurs in small headwater streams formerly surrounded by Atlantic Rainforest. The species has been collected in still water and fast-flowing hill-side streams, in clear, black or turbid water, over mud, sand and gravel bottoms.

Etymology. The specific name *circummontanus* (Latin, adjective, masculine, singular) is a combination of the word *circum*, meaning around, with the word *montanus*, meaning "of mountains". It is an allusion to the distribution of the species around the Serra da Mantiqueira and Serra do Mar mountain ranges, as well as associated highlands that dominate the Southeastern Region of Brazil.

Conservation status. *Phalloceros circummontanus* is widely distributed in six Brazillian states, with an estimated Extension of Occurrence (EOO) of 605,600 km². A substantial number of specimens recorded from numerous localities and collecting expeditions over the past two decades suggests that the species is not threatened, despite the extensive human occupation of southeastern Brazil. Based on these criteria, *P. circummontanus* is provisionally categorized as Least Concern (LC) according to the International Union for Conservation of Nature (IUCN, 2024).

Molecular delimitation. To validate the distinction of *Phalloceros circummontanus* from morphologically similar species, we compared DNA sequences of the mitochondrial CO1 gene from 34 individuals of *P. circummontanus* with 32 samples of *P. enneaktinos*, 17 samples of *P. harpagos*, and 18 samples of *P. titthos*. These sequences included samples from the type-localities and additional localities from the known distribution ranges of each species (Tab. **S2**). DNA-sequence comparisons confirmed that the four species are genetically distinct (Tab. 4). The unprecedented sequencing of four topotypes of *P. harpagos* resulted in a single reference haplotype (Genbank accessions: PQ279178, PQ279112, PQ279104, PQ279170), not related to haplotypes of *P. circummontanus* (Figs. **S5**, **S6**). The genetic divergence between *P. circummontanus* and *P. harpagos* is more than 6%, far exceeding accepted maximum levels of intraspecific divergence in fishes (Hebert *el al*., 2003a,b; Pereira *et al*., 2013). Additionally, the Refined Single Linkage delimitation method (Ratnasingham, Hebert, 2013) performed by the BOLD Systems (http://www.boldsystems.org/) assigned the four species to distinct Barcode Index Numbers (BIN). Specimens of *P. circummontanus* were assigned to BIN AAB5569, *P. enneaktinos* were assigned to BIN AAC0280, *P. harpagos* were assigned to BIN AFX6626, and specimens of *P. titthos* were assigned to BIN AAB5570.

TABLE 4 | Pairwise genetic distances for cytochrome oxidase c subunit 1 (CO1) gene between species pairs of *Phalloceros* calculated using a Kimura 2 Parameter model (below the diagonal). Intraspecific variation is shown in the diagonal. Variance estimates are shown above the diagonal and were obtained by a bootstrap procedure (100 replicates). N = number of samples of 655 nucleotides in the alignment.

Species	1	2	3	4
1 - *P. circummontanus* (N = 34)	0.0062	0.0109	0.0088	0.0103
2 - *P. enneaktinos* (N = 32)	0.0736	0.0045	0.0098	0.0101
3 - *P. harpagos* (N = 17)	0.0608	0.0563	0.0063	0.0093
4 - *P. titthos* (N = 18)	0.0674	0.0599	0.0595	0.0061

DISCUSSION

The original description of *Phalloceros harpagos* included a note on morphological and color variations among specimens from the La Plata system and coastal basins. The morphological evidence available at that time led its author to consider these variations as intraspecific, with the following justification: "At the moment, it is advisable to treat these differences as intraspecific variation, unless new evidences become available and allow the recognition of new diagnosable taxonomic entities" (Lucinda, 2008:138). Our comprehensive morphological and molecular analyses demonstrate that *P. harpagos* does not inhabit the upper rio Paraná basin and adjacent river basins as originally proposed and is genetically distinct from populations of those drainages at a level that fully justifies the recognition of those populations as a distinct species.

Phalloceros circummontanus was included in a previous phylogenetic analysis of the genus *Phalloceros* based on nuclear genomic data, where it was identified as "*P. harpagos* (Tietê)" (fig. 3 in Thomaz *et al*., 2019). According to the phylogenetic hyphothesis produced in that study, samples from the rio Tietê drainage are not related to samples of *P. harpagos* collected close to its type-locality in the rio Paraíba do Sul basin. The species from the Tietê drainage, here described as *P. circummontanus*, belongs to a clade that includes *P. titthos* and a putative undescribed species named "*P. harpagos* (PR-SP)" from the upper rio Iguaçu and upper rio Paranapanema drainages in the southeastern portion of the upper rio Paraná basin, and from adjacent costal basins (Oliveira *et al*., 2024). The presence of multiple species of the so-called "*P. harpagos* species complex" in the upper rio Paraná basin implied by the phylogenetic analysis advanced by Thomaz *et al*. (2019) was independently corroborated by four distinct methods of species delimitation based on CO1 gene sequencing (Oliveira *et al*., 2024). Our study provides a third independent dataset that further demonstrates that the species complex of upper rio Paraná basin contains undescribed species belonging to a monophyletic group that is not closely related to the populations of *P. harpagos* from the middle rio Paraíba do Sul basin.

Although the description of all species of the so-called *Phalloceros harpagos* complex (Oliveira *et al*., 2024) is beyond the scope of this study, it is possible to interpret the phylogenetic hypothesis from Thomaz' *et al*. (2019). According to that phylogenetic hypothesis, *P. circummontanus* (= "*P. harpagos* (Tietê)") is more closely related to the candidate species "*P. harpagos* (PR-SP)" from upper Iguaçu and to *P. tithos* than to *P. harpagos*. This finding prompts a reevaluation of the so-called "*P. harpagos* complex". Here, we exclude *P. harpagos* from this species complex and provisionally rename the set of upper Paraná populations as the *P. circummontanus* complex. The geographic limits of the complex are thus restricted to the upper rio Paraná basin and neighboring drainages, excluding tributaries of the middle rio Paraíba do Sul and coastal basins in the metropolitan region of Rio de Janeiro, which are inhabited by *P. harpagos.*

Oliveira *et al*. (2024) investigated the hidden diversity previously associated with *P. harpagos sensu* Lucinda (2008), identifying eight phylogroups. None of their samples corresponds to *P. harpagos* (BIN AFX6626; see Results). Instead, those phylogroups represent *P. circummontanus* (phylogroups 14, 15, and 16; BIN AAB5569), *P. enneaktinos* (phylogroup 7; BIN AAC0280), and three putative undescribed species: "*P. harpagos* (PR-SP)" (phylogroup 11; BIN ABY8844; Thomaz *et al*., 2019), *Phalloceros* sp. Macaé (phylogroup 1; BIN AAY4972; Souto-Santos *et al*., 2023a), and *Phalloceros* sp. Caputera (phylogroup 5; BIN ADM9037).

Phalloceros circummontanus was not recorded in syntopy with species of similar genital morphology, even within shared hydrographic basins. For instance, *P. circummontanus* and *P. harpagos* occur in different sections of the rio Paraíba do Sul basin, with *P. circummontanus* restricted to the upper rio Paraíba do Sul, and *P. harpagos*, to the middle section. Similarly, while *P. circummontanus*, *P. titthos*, and the putatively undescribed species related to *P. circummontanus* (Thomaz *et al*., 2019; Oliveira *et al*., 2024) inhabit the Iguaçu drainage (upper Paraná), the former is confined to the lower Iguaçu (below Iguaçu falls), and the latter two are restricted to the upper Iguaçu. Conversely, *P. circummontanus* and *P. harpagos* may occur in syntopy with species exhibiting divergent genital morphologies. In the rio Paraíba do Sul basin, *P. harpagos* is syntopic with *P. leptokeras*. In the upper Paraná basin *P. circummontanus* co-occurs with *P. reisi* in both the Tietê and Grande drainages. These syntopic species pairs have divergent genital morphologies, that may represent prezygotic reproductive barriers preventing hybridization (Jennings *et al*., 2023). The observed correlation between genital morphology and syntopy with congeneric species in *P. harpagos* and *P. circummontanus* suggests that genital divergence is a significant driver of speciation in *Phalloceros*. These findings underscore the importance of integrating morphological and molecular data into evolutionary and biogeographic studies of this genus.

In summary, our findings provide further evidence that integrative studies (Dayrat, 2005) combining morphological and molecular data constitute a powerful approach in resolving taxonomic uncertainties and elucidating cryptic diversity within species complexes (*e.g*., Reis *et al*., 2021). In this study, we present the first molecular characterization of *Phalloceros harpagos* using DNA sequences obtained from topotypical specimens, providing compelling evidence that the specimens from the upper Paraná and some coastal basins are not related to *P. harpagos.*

Comparative material examined. Brazil: *Phalloceros alessandrae*: MNRJ 40804, 39, 9.8–30.1 mm SL, topotypes. *Phalloceros anisophallos*: MNRJ 43253, 657, 7.7–42.5 mm SL, topotypes. *Phalloceros buckupi*: MCP 30472, 1, 19.3 mm SL, holotype. MNRJ 41236, 39, 16.2–35.1 mm SL. *Phalloceros caudimaculatus*: MNRJ 41089, 17, 16.4–30.2 mm SL. MNRJ 53012, 20, 7.8–32.8 mm SL. MNRJ 53339, 197, 15.6–29.0 mm SL. *Phalloceros elachistos*: MNRJ 41910, 51, 15.1–26.8 mm SL. *Phalloceros enneaktinos*: MNRJ 14847, 14, 11.7–32.8 mm SL, paratypes. MNRJ 20250, 28, 11.1–22.1 mm SL. MNRJ 20252, 44, 14.0–40.4 mm SL. MNRJ 23609, holotype, 24.2 mm SL. MNRJ 42910, 43, 7.5–29.8 mm SL. MNRJ 43245, 467, 12.7–31.5 mm SL, topotypes. *Phalloceros harpagos*: MNRJ 14745, 59, 13.5–37.8 mm SL, paratypes. MNRJ 23610, 26.9 mm SL, holotype. MNRJ 43595, 84/1, 13.5–31.7 mm SL, topotypes. *Phalloceros heptaktinos*: MNRJ 53319, 111, 14.5–33.9 mm SL, topotypes. *Phalloceros leptokeras*: MNRJ 43596, 284/2, 8.4–31.5 mm SL, topotypes. *Phalloceros leticiae*: MCP 30550, 2, 18.4–20.8 mm SL, paratypes. MCP 30551, 1, 16.2 mm SL, holotype. USNM 330339, 5/2, 14.3–17.6 mm SL, paratypes. *Phalloceros lucenorum*: MNRJ 40762, 32, 12.1–31.3 mm SL, topotypes. *Phalloceros malabarbai*: MNRJ 40909, 48, 10.5–37.9 mm SL. *Phalloceros maldonadoi*: MNRJ 41233, 108/4, 10.4–48.5 mm SL, paratypes. MNRJ 53606, 39.4 mm SL, holotype. MCP 54916, 40, 11.7–39.8 mm SL, paratypes. MZUSP 127010, 40, 9.9–47.3 mm SL, paratypes. UNT 21344, 40, 10.4–46.8 mm SL, paratypes. *Phalloceros megapolos*: MNRJ 40954, 261, 7.8–36.5 mm SL, topotypes. *Phalloceros mikrommatos*: MNRJ 50123, 36, 10.1–22.9 mm SL, topotypes. *Phalloceros ocellatus*: MNRJ 50126, 52, 10.3–25.6 mm SL, topotypes. *Phalloceros pellos*: MNRJ 40812, 199, 10.0–35.9 mm SL, topotypes. *Phalloceros reisi*: MNRJ 54524, 5, 24.1–36.1 mm SL. MNRJ 39228, 17, 13.5–31.8 mm SL. MNRJ 54856, 27, 13.7–34.8 mm SL. MNRJ 53997, 54, 13.8–40.9 mm SL. *Phalloceros spiloura*: MNRJ 55622, 29, 8.6–28.0 mm SL, topotypes. MNRJ 52571, 9, 14.5–24.2 mm SL. *Phalloceros titthos*:

MZUSP 43467, 21/1, 21.2–44.5 mm SL, paratypes. MNRJ 52359, 248, 13.0–33.8 mm SL. *Phalloceros uai*: MNRJ 43598, 388, 10.0–33.7 mm SL, topotypes.

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REFERENCES

- **Bell JR.** A simple way to treat PCR products prior to sequencing using ExoSAP-IT®. BioTechniques. 2008; 44(6):834. https://doi.org/10.2144/000112890
- **Dayrat B.** Towards integrative taxonomy. Biol J Linn Soc. 2005; 85(3):407–15. https://doi.org/10.1111/j.1095-8312.2005.00503.x

- **Gosline WA.** The sensory canals of the head in some Cyprinodont fishes, with particular reference to the genus *Fundulus*. Occ Pap Mus Zool Univ Mich. 1949; 519:1–17.
- **Hebert PDN, Cywinska A, Ball SL, deWaard JR.** Biological identifications through DNA barcodes. Proc R Soc B. 2003a; 270(1512):313–21. http://doi.org/10.1098/rspb.2002.2218
- **Hebert PDN, Ratnasingham S, deWaard JR.** Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proc R Soc B. 2003b; 270:596–99. http://doi.org/10.1098/rsbl.2003.0025
- **International Union for Conservation of Nature (IUCN). Standards and petitions subcommittee.** Guidelines for using the IUCN Red List categories and criteria. Version 16 [Internet]. Gland; 2024. Available from: https://www.iucnredlist.org/documents/RedListGuidelines.pdf
- **Jennings WB, Souto-Santos ICA, Buckup PA, Zandonà E.** Do mismatched genitalia promote mechanical isolation in syntopic species of *Phalloceros* fishes (Poeciliidae)? Zool J Linn Soc-Lond. 2023; 199(1):206–15. https://doi.org/10.1093/zoolinnean/zlad034
- **Kumar S, Stecher G, Li M, Knyaz C, Tamura K.** MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018; 35(6):1547–49. https://doi.org/10.1093/molbev/msy096
- **Leigh JW, Bryant D.** PopArt: full-feature software for haplotype network construction. Methods Ecol Evol. 2015; 6(9):1110–16. https://doi.org/10.1111/2041-210X.12410
- **Lucinda PHF.** Systematics of the genus *Cnesterodon* Garman, 1895 (Cyprinodontiformes, Poeciliidae, Poeciliinae). Neotrop Ichthyol. 2005; 3(2):259–70. https://doi.org/10.1590/S1679-62252005000200003
- **Lucinda PHF.** Systematics and biogeography of the genus *Phalloceros* Eigenmann, 1907 (Cyprinodontiformes: Poeciliidae: Poeciliinae), with the description of twenty-one new species. Neotrop Ichthyol. 2008; 6(2):113–58. https://doi.org/10.1590/S1679-62252008000200001
- **Lucinda PHF, Reis RE.** Systematics of the subfamily Poeciliinae Bonaparte (Cyprinodontiformes: Poeciliidae), with an emphasis on the tribe Cnesterodontini Hubbs. Neotrop Ichthyol. 2005; 3(1):1–60. https://doi.org/10.1590/S1679-62252005000100001
- **Miller SA, Dykes DD, Polesky HF.** A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988; 16(3):1215. https://doi.org/10.1093/nar/16.3.1215
- **Mishler BD, Theriot EC.** The phylogenetic species concept (*sensu* Mishler and Theriot): monophyly, apomorphy and phylogenetic species concept. In: Wheeler QD, Meier R, editors. Species concepts and phylogenetic theory. New York: Columbia University Press; 2000. p.45–54.
- **Oliveira IJ, Diamante NA, Fabrin TMC, Frota A, Graça WJ, Oliveira AV, Prioli SMAP, Prioli AJ.** Hidden diversity and evolutionary diversification in *Phalloceros harpagos* Lucinda (Cyprinodontiformes: Poeciliidae). Ecol Freshw Fish. 2024; 33(1):e12741. https://doi.org/10.1111/eff.12741
- **Pereira LHG, Hanner R, Foresti F, Oliveira C.** Can DNA barcoding accurately discriminate megadiverse Neotropical freshwater fish fauna. BMC Genet. 2013; 14:20. https://doi.org/10.1186/1471-2156-14-20
- **Reis RE, Vieira F, Pereira EHL.** A new species of the loricariid catfish genus *Loricariichthys* (Teleostei: Siluriformes) from eastern Brazil. Ichthyol Herpetol. 2021; 109(2):557–66. https://doi.org/10.1643/i2020013
- **Rosen DE, Gordon M.** Functional anatomy and evolution of male genitalia in Poeciliid fishes. Zoologica. 1953; 38:1–47.
- **Sabaj MH.** Codes for natural history collections in ichthyology and herpetology. Copeia. 2020; 108(3):593–669. https://doi.org/10.1643/ASIHCODONS2020
- **Souto-Santos ICA, Jennings WB, Buckup PA.** Testing palaeodrainage hypotheses in south-eastern Brazil: phylogeography of the sinistral livebearer fish of the genus *Phalloceros* (Cyprinodontiformes: Poeciliidae). Zool J Linn Soc-Lond. 2023a; 197(2):514–31. https://doi.org/10.1093/zoolinnean/zlac030

- **Souto-Santos ICA, Lucinda PHF, Buckup PA.** Bilateral genital asymmetry in livebearer fishes of the genus *Phalloceros* Eigenmann, 1907, with description of a new species from coastal drainages of southern Brazil (Cyprinodontiformes: Poeciliidae). J Fish Biol. 2023b; 103(1):91–102. https://doi.org/10.1111/jfb.15410
- **Taylor WR, Van Dyke GC.** Revised procedures for staining and clearing small fishes and other vertebrates for bone and cartilage study. Cybium. 1985; 9(2):107–19. https://doi.org/10.26028/cybium/1985-92-001
- **Templeton AR, Crandall KA, Sing CF.** A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics. 1992; 132(2):619–33. https://doi.org/10.1093/genetics/132.2.619
- **Thomaz AT, Carvalho TP, Malabarba LR, Knowles LL.** Geographic distributions, phenotypes, and phylogenetic relationships of *Phalloceros* (Cyprinodontiformes: Poeciliidae): insights about diversification among sympatric species pools. Mol Phylogenet Evol. 2019; 132:265–74. https://doi.org/10.1016/j.ympev.2018.12.008
- **Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ.** W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res. 2016; 44(1):232–35. https://doi.org/10.1093/nar/gkw256
- **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(1462):1847–57. https://doi.org/10.1098/rstb.2005.1716

AUTHORS' CONTRIBUTION

Igor C. A. Souto-Santos: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing-original draft.

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Dahiana Arcila: Funding acquisition, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing-review and editing.

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ETHICAL STATEMENT

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COMPETING INTERESTS

The authors declare no competing interests.

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