Zoological Journal of the Linnean Society, 2023, XX, 1–10
https://doi.org/10.1093/zoolinnean/zlad034
Advance access publication 29 June 2023
Original Article

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Do mismatched genitalia promote mechanical isolation in syntopic species of *Phalloceros* fishes (Poeciliidae)?

W. Bryan Jennings[1,2,3,*,‡], Igor C.A. Souto-Santos[2,‡], Paulo A. Buckup[2], Eugenia Zandonà[1]

[1]Departamento de Ecologia, Programa de Pós-Graduação em Ecologia e Evolução, Universidade do Estado do Rio de Janeiro, 20550-013 Rio de Janeiro, RJ, Brazil
[2]Museu Nacional, Departamento de Vertebrados, Universidade Federal do Rio de Janeiro, Quinta da Boa Vista, 20490-040 Rio de Janeiro, RJ, Brazil
[3]Department of Evolution, Ecology, and Organismic Biology, University of California, Riverside, Riverside, CA 92521, USA

‡These authors contributed equally to this work.

[*]Corresponding author. Departamento de Ecologia, Programa de Pós-Graduação em Ecologia e Evolução, Universidade do Estado do Rio de Janeiro, 20550-013 Rio de Janeiro, RJ, Brazil. E-mail: bryan.jennings@ucr.edu

ABSTRACT

The elucidation of mechanisms responsible for the reproductive isolation of species is a fundamental part of speciation research. South American poeciliid fishes in the genus *Phalloceros* represent a promising system in which to study the evolution of reproductive barriers between closely related species. *Phalloceros* are often found in sympatry with non-sister congeners, and most such species pairs have morphologically divergent female and male genitalia. In recent studies, it has been hypothesized that mismatched genitalia between co-occurring *Phalloceros* species might act as mechanical barriers to prevent hybridization and might help to explain the diversification of this group. However, this idea has not yet been evaluated empirically with genetic data. Here, we tested this hypothesis using morphological data in conjunction with mitochondrial and nuclear DNA sequence data. Our study focused on *Phalloceros anisophallos* and *Phalloceros leptokeras*, two non-sister species that have mismatched genitalia and that occur together in at least four rivers in south-eastern Brazil. Despite the prevalence of hybridization between closely related non-sister species of animals, especially fish, our phylogenetically-based results detected no evidence of mitonuclear discordance and (hence historical hybridization) between the two focal species. Our findings are therefore consistent with the hypothesis that mismatched genitalia prevent hybridization between sympatric species of *Phalloceros* fishes.

Keywords: Atlantic Rainforest; low genetic diversity; molecular phylogenetics; non-sister species; reproductive character displacement

INTRODUCTION

Mechanical isolation caused by incompatibility between the reproductive structures of two closely related species is presumed to prevent, or at least significantly reduce, the frequency of fertilization events between them (Dufour 1844; Paulson 1974; Sota and Kubota 1998; Coyne and Orr 2004). Indeed, mechanical isolation barriers, especially in animals, might be important in promoting speciation and in maintaining species boundaries thereafter (Eberhard 1985; Coyne and Orr 2004). According to sexual selection theory, mechanical isolation can be achieved via two non-mutually exclusive ways: (1) 'tactile isolation' or selection on male genitalia caused by females that prefer to mate with conspecific males while avoiding copulation with heterospecific males (Eberhard 1985; Arnqvist and Danielsson 1999; Bernstein and Bernstein 2002; Coyne and Orr 2004); and (2) 'lock-and-key isolation' or co-evolution of conspecific male and female genitalia, which might lead to failed mating attempts between heterospecific individuals owing to 'mismatched' genitalia (Coyne and Orr 2004; Anderson and Langerhans 2015). Although there are still few well-established cases of lock-and-key isolation in animals, future research might reveal far more cases of this form of reproductive isolation, and thus its relevance to speciation and the evolutionary diversification of lineages (Coyne and Orr 2004; Anderson and Langerhans 2015). One study system that shows great promise in this regard is the genus *Phalloceros* Eigenmann, 1907 (Thomaz *et al.* 2019), a group of freshwater fish that is endemic to rivers and streams in south-eastern South America (Lucinda 2008; Reznick *et al.* 2017).

Species of *Phalloceros* have been the subjects in studies of life-history traits (Arias and Reznick 2000; Almeida-Silva and Mazzoni 2014; Gorini-Pacheco *et al.* 2018; Zandonà *et al.* 2021),

Received 2 December 2022; revised 2 April 2023; accepted 5 April 2023

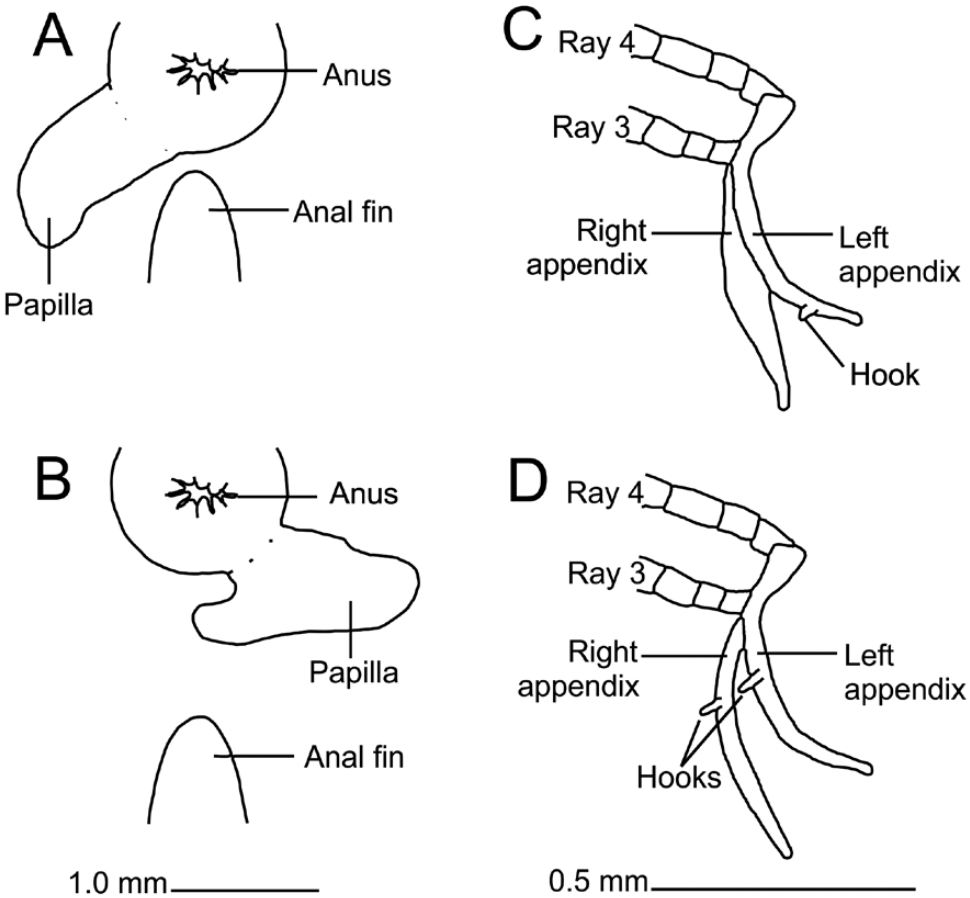

Figure 1. Interspecific differences in genitalia of mature individuals of *Phalloceros anisophallos* and *Phalloceros leptokeras*. A, B, ventral views of female urogenital papillae of *P. anisophallos* (A) and *P. leptokeras* (B). Notice that the papilla is turned to the right from the perspective of the fish and points somewhat distally towards the tail in *P. anisophallos,* and is turned to the left and points somewhat laterally in *P. leptokeras*. C, D, lateral view of the distal portion of the gonopodia with the skin removed of *P. anisophallos* (C) and *P. leptokeras* (D). These schematic drawings simply depict the generalized morphologies of the genitalia for each species and thus do not reflect any single museum specimen.

ecology (Mazzoni *et al.* 2011), population genetics (Amaral *et al.* 2015), and speciation and biogeography (Lucinda and Reis 2005; Souto-Santos *et al.* 2019, 2023; Thomaz *et al.* 2019). A major surge in *Phalloceros* research started after Lucinda (2008) formally split the once monotypic genus (i.e. *Phalloceros caudimaculatus* Hensel 1868) into 22 different species, based largely on interpopulational variation in the external reproductive structures of mature adult females and males, called the urogenital papilla and gonopodia, respectively. The female papilla is a fleshy projection located between the anus and the origin of the anal fin, while the male gonopodium is a phallus-like structure derived from anal-fin rays (Souto-Santos *et al.* 2019). Lucinda used the spatial orientation of papillae (i.e. turned left, pointed back along the midline or turned right) to help differentiate species of *Phalloceros*. To distinguish *Phalloceros* species using variation in male gonopodia, Lucinda (2008) described species-specific characteristics of the gonopodial appendices, which are paired, fork-like projections at the tip of the gonopodium (Souto-Santos *et al.* 2019).

Species of *Phalloceros* are frequently found in sympatry (i.e. same river or stream) and even in syntopy (i.e. same collecting sites) with each other (Lucinda 2008; Souto-Santos *et al.* 2019; Thomaz *et al.* 2019). In a recent phylogenetic study of *Phalloceros*, Thomaz *et al.* (2019) noted that most of the species in this genus were sympatric or syntopic with at least one congener. In another study, Souto-Santos *et al.* (2019) discovered that *Phalloceros anisophallos* Lucinda, 2008 was syntopic with *Phalloceros aspilos* Lucinda, 2008 and *Phalloceros tupinamba* Lucinda, 2008 (both are now referred to as *Phalloceros leptokeras*; Souto-Santos *et al.* 2023) and *P. harpagos* Lucinda, 2008. In nearly all known cases of sympatric *Phalloceros* species, robust phylogenetic evidence showed that the co-occurring species are non-sister species (Thomaz *et al.* 2019). These findings led Thomaz *et al.* (2019) to suggest that *Phalloceros* had diversified via allopatric speciation followed by secondary contact.

Syntopic species in this genus seem to have genitalia that are markedly different from each other. For example, *P. anisophallos* and *P. leptokeras*, which are non-sister species (Lucinda 2008; Thomaz *et al.* 2019) and are syntopic at some sites, have mismatched genitalia: mature females of *P. anisophallos* have right-turned papillae (Fig. 1A), whereas females of *P. leptokeras* have left-turned papillae (Fig. 1B); and mature males of *P. anisophallos* have a gonopodial tip with a hook on only the left appendix and a right appendix that is wider than the left one (Fig. 1C), whereas males of *P. leptokeras* have a hook protruding from both appendices (Fig. 1D) (Souto-Santos *et al.* 2019). Thomaz *et al.* (2019) pointed out this same general pattern for other sympatric species pairs of *Phalloceros*. These observations led Souto-Santos *et al.*

(2019) and Thomaz *et al.* (2019) independently to suggest that mismatched genitalia between syntopic species of *Phalloceros* might act as mechanical barriers to prevent heterospecific individuals from hybridizing with each other. Building on this idea, Thomaz *et al.* (2019) speculated that reproductive character displacement might have played a role in diversification of the group. However, a key hypothesis of this model of *Phalloceros* diversification holds that syntopic species are unable to hybridize with each other owing to their presumably incompatible genitalia.

One way to detect hybridization between two closely related but non-sister species that are sympatric is to search for topological incongruence between reconstructed mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) gene trees; a phenomenon known as mitonuclear discordance (Toews and Brelsford 2012). Indeed, clear evidence of introgression comes from studies that show two sympatric and genetically/morphologically divergent species sharing the same geographically localized mtDNA haplotypes, especially if original mtDNA haplotypes of each species are known (Funk and Omland 2003). Although there are several possible causes of conflicts among gene trees, or between gene trees and the presumed species tree [i.e. introgression, lateral gene transfer, recombination (nuclear genes only) and incomplete lineage sorting; Moore 1995; Maddison 1997; Degnan and Rosenberg 2009], such incongruence is thought commonly to be the result of introgression (e.g. Toews and Brelsford 2012; Sarver *et al.* 2017). Mitonuclear discordance is a widespread phenomenon among vertebrates, including fishes (Toews and Brelsford 2012), and thus comparisons of the topologies of mtDNA and nDNA gene trees for individuals representing two closely related but non-sister species can reveal evidence of past hybridization events. Here, we use this phylogenetically based approach to test the hypothesis that syntopic *P. anisophallos* and *P. leptokeras* are reproductively isolated from each other.

MATERIALS AND METHODS

Study species and focal populations

In this study, we focused on the populations of *P. anisophallos* and *P. leptokeras* in the Parati-Mirim River (23°14′24.36″S, 44°38′55.13″W). These two species are distributed in the coastal and near-coastal drainages of the Atlantic Rainforest ecosystem in south-eastern Brazil (Lucinda 2008; Souto-Santos *et al.* 2019, 2023; Thomaz *et al.* 2019). *Phalloceros anisophallos* is known from streams and rivers in the coastal side of the Serra do Mar region and on the island of Ilha Grande in the state of Rio de Janeiro (Lucinda 2008; Almeida-Silva and Mazzoni 2014; Souto-Santos *et al.* 2019), while *P. leptokeras* has a broadly overlapping distribution, being found along the Serra do Mar in the states of Rio de Janeiro and São Paulo (Souto-Santos *et al.* 2019, 2023). According to the literature, both have been collected at the same sites in the Parati-Mirim and Japuíba rivers and thus are syntopic with each other in those rivers (see maps Souto-Santos *et al.* 2019, 2023).

Species identifications based on morphological data

We defined three age classes: 'juveniles' had undeveloped genitalia and thus could not be sexed; 'subadults' had underdeveloped genitalia but could be assigned as being male or female; and 'adults' had fully developed genitalia. We used two different approaches for species identification (species ID) using morphological data: genitalia for adult individuals and lateral body spots for juveniles and subadults. For adult females, we used the orientation of the urogenital papilla to assign species IDs (see Fig. 1A, B), and for sexually mature males we relied on the morphology of the gonopodial appendices (see Fig. 1C, D). Given that we could not identify juvenile and subadult specimens using genital characters, we could only provisionally use the presence or absence of a dark vertically oriented bilateral bar or 'spot' on each side to assign individuals to *P. anisophallos* or *P. leptokeras*, respectively. Although Lucinda (2008) had found the presence/absence of lateral spots to be a good indicator of species for individuals belonging to *P. anisophallos* (with spots) or *P. aspilos* (without spots; currently named *P. leptokeras*; see Souto-Santos *et al.* 2023), this character was later shown to be unreliable (Souto-Santos *et al.* 2023: table S3) for identifying individuals of *P. leptokeras* from the Parati-Mirim River. However, it remains to be seen whether this character can be used to identify reliably individuals of *P. anisophallos* from the Parati-Mirim River or elsewhere. All specimens were sourced from the Ichthyology Collection at the Museu Nacional (MNRJ), Universidade Federal do Rio de Janeiro. Catalogue numbers of examined specimens can be found in the Supporting Information (Table S1).

Molecular data

We obtained two DNA sequence datasets: one consisted of a 656-bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene (i.e. animal barcode of life marker; Hebert *et al.* 2003a, b), while the other was composed of a 602-bp portion of the nuclear recombination activation gene 1 (*RAG1*). We chose these two markers because they have species-specific haplotypes for *P. anisophallos* and *P. leptokeras* (P. A. Buckup, I. C. A. Souto-Santos and W. B. Jennings, **unpublished data**). To establish the correct haplotype sequences for each species in our mtDNA and nDNA gene trees, we included reference conspecific sequences for additional river drainages. These included: (1) *P. anisophallos* specimens from places where *P. leptokeras* is not known to occur (i.e. Ambrósio River, Caputera River, Jurumirum River, Perequê Açu River and the São Roque River); and (2) *P. leptokeras* specimens from places where *P. anisophallos* does not occur [i.e. Grande River, Indaiá River, Itamambuca River, Macacu River, Mazomba River, Paraíba do Sul River (Paquequer and Pocinhos), Puruba River and Quiririm River]. Samples from closely related species were also included as references, based on a previously existing phylogenetic hypothesis for *Phalloceros*. We therefore added *Phalloceros elachistos* Lucinda, 2008 (Sales *et al.* 2018), *Phalloceros enneaktinos* Lucinda, 2008, *Phalloceros mikrommatos* Lucinda, 2008 (Souto-Santos *et al.* 2023) and *Phalloceros ocellatus* Lucinda, 2008 (Souto-Santos *et al.* 2023), which are expected to be more closely allied with *P. leptokeras* than with *P. anisophallos*, whereas *P. caudimaculatus* (GenBank MK660681), *Phalloceros malabarbai* Lucinda, 2008 (Jennings *et al.* 2019), *Phalloceros reisi* Lucinda, 2008 (Pereira *et al.* 2011) and *Phalloceros spiloura* Lucinda, 2008 (GenBank MK660682) are expected to be closer to *P. anisophallos* than to *P. leptokeras* (see Thomaz *et al.* 2019: fig. 3). To root our *COI* and *RAG1* gene trees, we included the outgroup species *Cnesterodon* *decemmaculatus* Jenyns, 1842 (Rosso *et al.* 2012) and *Poecilia vivipara* Bloch & Schneider, 1801 (Souto-Santos *et al.* 2023). The sources of the DNA sequences used in this study are listed in the Supporting Information (Table S1).

To obtain new DNA sequences for this study, we dissected tissue samples by using a sterile scalpel blade to remove a fillet from one side of each specimen. We used the saline extraction method (Miller *et al.* 1988) to extract genomic DNA from ~5 mg of tissues preserved in 99% ethanol. Next, we carried out standard PCRs by using 10–100 ng of template DNA in 25 μL reactions together with the universal fish *COI* primers FISH-F6 5′-TACYAAYCACAAAGAYATTGGCA-3′ and FISH-R7 5′-TARACTTCTGGRTGDCCRAAGAAYCA-3′ (Jennings *et al.* 2019) or the fish-specific *RAG1* primers RAG1PF3 5′-CACTGCGACATAGGCAATGC-3′ and RAG1PFR3 5′-GGTGTAGAGCCAGTGGTGTT-3′ (Souto-Santos *et al.* 2023). After we confirmed the presence of DNA bands of the correct size in 2% agarose gels, we purified the PCR products using the 20% polyethylene glycol method (Jennings 2017), then sequenced them in both directions on an ABI automated sequencer (Applied Biosystems).

Sequence analyses

We used the software FinchTV (Geospiza, Seattle, WA, USA) to carry out manual checking of the quality of the raw sequence data by examining forward and reverse chromatogram files for each sequenced PCR product, a process that allowed us to correct occasional ambiguous base calls (for a description of the method, see Jennings, 2017). We then used the program Se-AL (Rambaut 2007) to construct contig sequences manually, based on the forward and reverse sequences for each PCR product. We aligned each set of sequences by eye before verifying that they represented orthologous gene copies rather than mitochondrial or nuclear pseudogenes (i.e. we ensured that the sequences did not contain indels or premature stop codons; Jennings 2017). The COI and RAG1 multiple sequence alignment files can be found in Supplementary Information documents (Files S1 and S2).

A phylogenetic analysis was conducted on each multiple sequence alignment separately using the program MrBayes (Ronquist *et al.* 2012). Each run implemented an HKY+Γ substitution model and consisted of two million generations, with a burn-in fraction set to 0.5. Convergence was deemed to have occurred after the average SD of tree scores became < 0.01.

Analysing the extent of syntopy in *Phalloceros*

To determine how pervasive syntopy is in *Phalloceros*, we compiled all known records of syntopy using literature sources (Lucinda 2008; Souto-Santos *et al.* 2019; Thomaz *et al.* 2019) and from specimens in the Ichthyological Collection at the MNRJ. We retained only records that met our stringent criteria for syntopy, as follows: (1) two or more species of *Phalloceros* are considered syntopic if they were collected at the same locality and at the same time; and (2) all records of syntopy are based on vouchered museum specimens. For each verified case of syntopy, we recorded the names of coexisting species, the orientation of the adult female papilla in each species (i.e. right, left or straight), the presence of adult male gonopodial hooks (i.e. a hook on each appendix or 'both hooks', a hook only on the left appendix or 'left hook', and 'no hooks'), the shape of the gonopodium (i.e. symmetrical or asymmetrical), the collection locality including GPS coordinates, the museum registration number for each voucher specimen, and the source of the data.

Analysis of the degree of genital mismatch in validated syntopic pairs

Although it is known that many sympatric, and perhaps syntopic, species of *Phalloceros* exhibit interspecific differences in adult female and male genitalia, it is not yet known whether these mismatches can inhibit hybridization between coexisting congeners in this genus. Moreover, given that genital morphology between a pair of syntopic species can be mismatched in multiple different ways (i.e. by papillae, by the number of gonopodial hooks and by gonopodial shape), it is conceivable that these mismatch variants vary in their efficacy to prevent cross-species fertilization.

Seven types of interspecific mismatch were recognized: (1) 'papillae only' means that papillae in adult females were oriented differently between coexisting species, but all other genital characters were invariant between those species; (2) 'hooks only' signifies that gonopodial hooks differed between coexisting species, but other genital characters were invariant between those species; (3) 'shape only' refers to two coexisting species that differed from each other only by the shapes of adult male gonopodia; (4) 'papillae + hooks' means that two syntopic species differed from each other in terms of their papillae and gonopodial hooks but not in gonopodial shape; (5) 'papillae + shape' refers to syntopic species that showed interspecific differences in the orientation of papillae and gonopodial shape, but not in gonopodial hooks; (6) 'hooks + shape' characterizes cases in which syntopic species differed from each other by their gonopodial hooks and shapes but not in papillae; and (7) 'papillae + hooks + shape' means that two syntopic species exhibited interspecific differences in all three genital characters. Given that, presumably, the degree of mismatch increases with the number of mismatched characters and might therefore be correlated with degrees of mechanical isolation between syntopic species, we defined three broad mismatch categories: one-character mismatch (lowest degree of mismatch); two-character mismatch (medium degree of mismatch); and three-character mismatch (highest degree of mismatch).

RESULTS

Our inferred mtDNA gene tree contains two haplotype clades that largely reflect the species IDs of our samples based on morphological criteria (Fig. 2A). Within the '*P. leptokeras*' clade, we identify six specimens from the Parati-Mirim River as *P. leptokeras* using morphological characters that agree with the morphology-based species names of conspecific specimens from other localities (Fig. 2A). Species IDs for five of these specimens are based on genital characters, while one juvenile is identified provisionally, using the spot/no spot criterion (Supporting Information, Table S2). In contrast, two other specimens from the Parati-Mirim River are identified morphologically as *P. anisophallos*, but their mtDNA haplotypes are nested within the *P. leptokeras* clade (Fig. 2A). One of these incorrectly

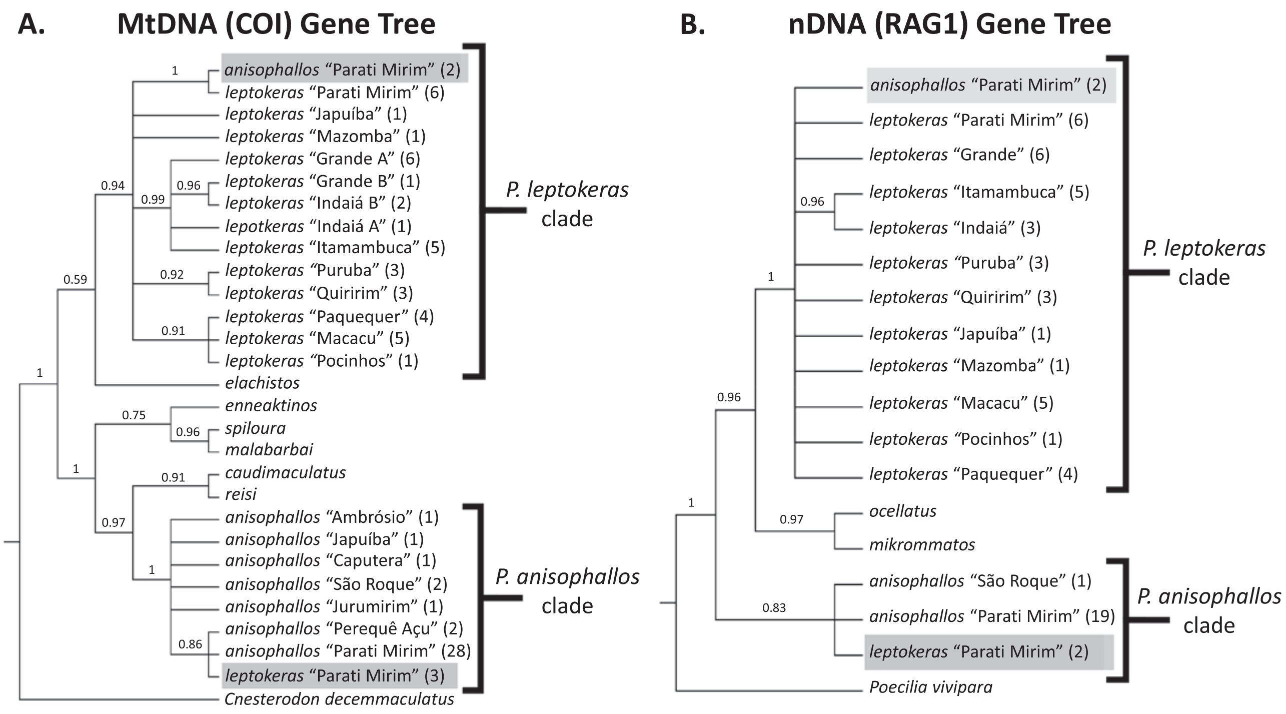

Figure 2. Bayesian mitochondrial (mtDNA) and nuclear (nDNA) gene trees, showing the inferred relationships among haplotypes of *Phalloceros anisophallos* and *Phalloceros leptokeras* from the Parati-Mirim River and other localities. A, mtDNA tree for the *COI* sequences. B, nDNA tree for the *RAG1* sequences. Shaded names refer to samples misidentified based on morphological criteria (see Materials and Methods). Numbers in parentheses represent the number of identical haplotypes. Other species of *Phalloceros* were included as reference samples, and *Cnesterodon decemmaculatus* and *Poecilia vivipara* were used to root the trees. *Phalloceros anisophallos* and *Phalloceros leptokeras* are sympatric in the Parati-Mirim and Japuíba rivers. São Roque and Paquequer correspond to the type localities of *P. anisophallos* and *P. leptokeras*, respectively (Lucinda 2008). Numbers above each branch are the estimated Bayesian posterior probabilities.

identified specimens is an adult female (MNTI 14559) that had been identified using genitalia, while the other is a subadult male (MNTI 14562) that was identified tentatively using the spots/no spots criterion (Supporting Information, Table S2).

We obtain similar results for the '*P. anisophallos* clade', in that the vast majority (28 of 31) of the specimens from the Parati-Mirim River that are identified morphologically as *P. anisophallos* cluster with the reference samples for this species to form the *P. anisophallos* mtDNA haplotype clade (Fig. 2A). Of the 28 samples whose morphological IDs match their mtDNA-based IDs, 24 are identified using characters of the genitalia, while seven immature individuals are identified provisionally using the spot/no spot criterion (Supporting Information, Table S2). In contrast, three other subadult/juvenile specimens (MNTIs 10607, 14533 and 14548) are identified provisionally as *P. leptokeras* based on their spotless conditions, although their mtDNA haplotypes suggest that they are instead *P. anisophallos* (Fig. 2A; Supporting Information, Table S2).

Our nDNA gene tree contains *P. leptokeras* and *P. anisophallos* clades that are comparable to those found in the mtDNA tree (Fig. 2B; Supporting Information, Table S2). Within the nDNA *P. leptokeras* clade (Fig. 2B), the two samples from the Parati-Mirim River that are identified morphologically as *P. anisophallos* (MNTIs 14559 and 14562) are the same two samples that are identified as *P. anisophallos* in the mtDNA *P. leptokeras* clade (Fig. 2B; Supporting Information, Table S2). In the former case, the adult female specimen (MNTI 14559) has a right-turning papilla, suggesting that it is *P. anisophallos*, while in the latter case the subadult individual (MNTI 14562) exhibits a lateral spot on both sides, also suggesting that it is *P. anisophallos* (Supporting Information, Table S2). Likewise, in the nDNA *P. anisophallos* clade, the three samples from the Parati-Mirim River that are identified as *P. leptokeras* based on their morphological characteristics (MNTIs 10607, 14533 and 14548) are the same samples that are identified as *P. leptokeras* in the mtDNA *P. anisophallos* clade (Fig. 2A, B; Supporting Information, Table S2). Given that the topologies of both gene trees agree with each other, we find no evidence of mitonuclear discordance, hence the morphological IDs of these five specimens must be in error.

To understand the basis of the species ID errors for the five specimens, we re-examined the external morphology of these specimens and compared them with the other specimens in our sample. One of the five incorrectly identified specimens was an adult female (MNTI 14559). Although the papilla of this specimen turns rightwards, and thus suggests to us that it is *P. anisophallos*, both mtDNA and nDNA sequences indicate that it is *P. leptokeras* (Fig. 3; Supporting Information, Table S2). Upon re-examination of this adult female, we discovered that its papilla has two other defining characteristics that suggest it is *P. leptokeras*. First, its papilla is pointed in a latero-anterior direction, as is seen in archetypal *P. leptokeras* individuals (compare MNTI 10684, 10684 and 10680 in Fig. 3), rather than in a distal direction, as is seen in typical *P. anisophallos* females (see MNTI 14553 and 10679 in Fig. 3). Second, the papilla of MNTI 14559 exhibits a fleshy process on its anterior side (see MNTI 14559 in Fig. 3). This fleshy process is a unique characteristic of

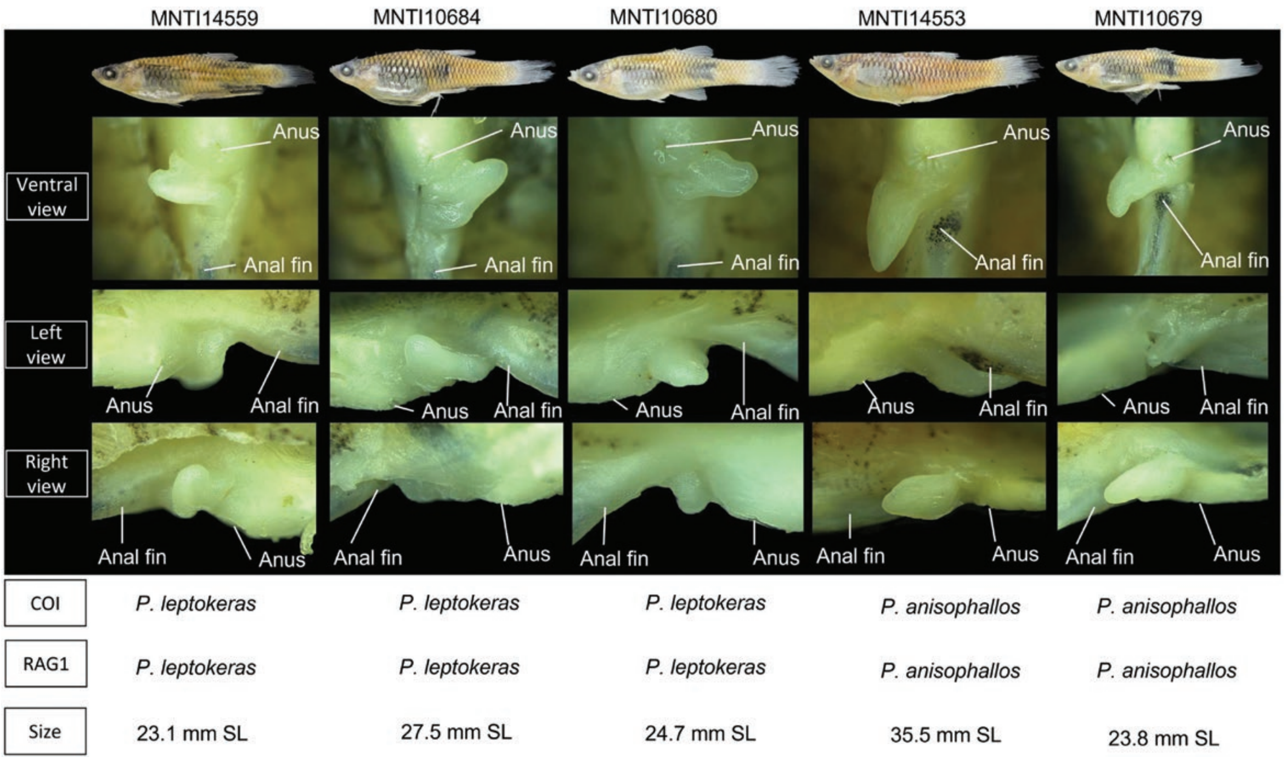

Figure 3. Among-individual variation in the presence/absence of dark lateral spots and the morphology of adult female papillae in *Phalloceros anisophallos* and *Phalloceros leptokeras* from the Parati-Mirim River in relationship to molecular barcode information. The barcode sequences for each specimen are listed at the bottom and correspond to mitochondrial (*COI*) and nuclear (*RAG1*) DNA haplotypes. Based on the orientation of genital papillae, these specimens were initially identified as follows: *P. anisophallos* = MNTI 14559, 14553 and 10679; and *P. leptokeras* = MNTI 10684 and 10680. Note that morphologically and molecularly based species identifications for individual MNTI 14559 conflicted with each other, probably owing to an apparently aberrant papilla (see main text for more discussion). The size of the specimens corresponds to the standard length (SL), measured with digital callipers from the tip of the snout to the posterior margin of the hypural plate.

P. leptokeras (Souto-Santos *et al.* 2023). We therefore conclude that individual MNTI 14559 is *P. leptokeras* with an aberrantly shaped papilla; that is, its papilla has the correct anatomy for *P. leptokeras*, but points in the wrong direction.

The other four mis-identified specimens were juveniles and subadults, which renders the practice of using the spot/no spot criterion to identify these species questionable. Indeed, one individual (MNTI 14562) has a spot on both sides and thus leads us initially to identify it as *P. anisophallos*, but both molecular barcode sequences indicate that it is instead *P. leptokeras* (Supporting Information, Table S2). The other three misidentified individuals (MNTIs 10607, 14533 and 14548) are all spotless, which suggests that they are *P. leptokeras*, but our molecular data instead identifies them as *P. anisophallos* (Supporting Information, Table S2). Assuming that the molecular barcodes provide accurate species ID results, four of the nine immature (two juvenile and seven subadult) specimens from the Parati-Mirim River were mis-identified using the presence/absence of lateral spot criterion (Supporting Information, Table S2).

Additional support for the hypothesis that the presence/absence of spots cannot be used to identify specimens belonging to these two species comes from results of a broader analysis of spotting patterns in our sample of specimens from the Parati-Mirim River. This analysis reveals substantial among-individual variation in the presence or absence of a dark lateral spot (Table 1). For example, we find that 57% and 72% of the *P. leptokeras* and *P. anisophallos* specimens, respectively, are spotless (Table 1).

Our scrutiny of primary literature sources and of specimens at the MNRJ revealed 32 instances of syntopy in *Phalloceros*, involving ≥ 14 species in this genus (Supporting Information, Table S3). Furthermore, there appeared to be a maximum of two coexisting species of *Phalloceros* at each collecting site. In all cases, the genital morphologies of adults belonging to each syntopic pair exhibited some degree of interspecific mismatch (Supporting Information, Table S3). From these data, we identified 17 unique syntopic species pairs: eight pairs showed relatively low mismatch, three pairs exhibited a medium level of mismatch, and six other pairs showed the highest level of mismatch (Table 2).

DISCUSSION

No evidence of mitochondrial introgression

The subjects of the present study, *P. anisophallos* and *P. leptokeras*, are ideal for evaluating the evolutionary significance of mismatched genitalia in *Phalloceros* because they can be found coexisting at the same sites in at least four rivers (Supporting Information, Table S3) and they show interspecific differences in genital morphology (Figs 1, 3; see also Lucinda 2008). Our mtDNA and nDNA gene trees showed no mitonuclear discordance, and therefore our results are consistent with our

Table 1. Presence of dark lateral spots on individuals of *Phalloceros anisophallos* and *Phalloceros leptokeras* from the Parati-Mirim River.

Molecular identity	Lateral spots	Number of specimens
Phalloceros anisophallos	Left side only	3[a]
P. anisophallos	Right side only	0
P. anisophallos	Left and right sides	6
P. anisophallos	No spots	23
Phalloceros leptokeras	Left side only	0
P. leptokeras	Right side only	1
P. leptokeras	Left and right sides	2
P. leptokeras	No spots	4

[a] Two of the three individuals of *Phalloceros anisophallos* that had a spot on the left side had their right sides formerly dissected, hence the presence of a spot on that side could not be assessed.

Table 2. Relative degree of mismatch in characters of the genitalia between syntopic species of *Phalloceros*.

Genital character	Number of pairs	Mismatch	Syntopic species pairs
Papillae only	4	Low	*P. alessandre–P. titthos, P. enneaktinos–P. leptokeras, P. harpagos–P. leptokeras, P. megapolos–P. titthos*
Hooks only	4	Low	*P. buckupi–P. maldonadoi, P. caudimaculatus–P. titthos, P. malabarbai–P. spiloura, P. megapolos–P. spiloura*
Shape only	0	Low	None
Papillae + hooks	1	Medium	*P. buckupi–P. titthos*
Papillae + shape	0	Medium	None
Hooks + shape	2	Medium	*P. alessandre–P. pellos, P. megapolos–P. pellos*
Papillae + hooks + shape	6	High	*P. anisophallos–P. enneaktinos, P. anisophallos–P. harpagos, P. anisophallos–P. leptokeras, P. lucenorum–P. titthos, P. pellos–P. titthos, P. reisi–P. titthos*

'Papillae only', 'hooks only' and 'shape only' each refer to cases in which the adults of a syntopic species pair differ in only one genital character ('low' degree of mismatch). 'Papillae + hook', 'papillae + shape' and 'hooks + shape' describe cases whereby the adults of a syntopic species pair exhibit interspecific differences in two genital traits only ('medium' degree of mismatch). 'Papillae + hooks + shape' refers to cases whereby the adults of two syntopic species exhibit interspecific differences in all three genital traits ('high' degree of mismatch). Data are from the Supporting Information (Table S3). Note that *P. maldonadoi* is a newly described species of *Phalloceros* (see Souto-Santos et al. 2023).

expectation that these syntopic species cannot interbreed with each other, possibly owing to their mismatched genitalia. To our knowledge, this is the first empirical test, using molecular data, of the hypothesis that syntopic species of *Phalloceros* having mismatched genitalia cannot interbreed with each other.

Before attempting to detect cases of mitonuclear discordance between mtDNA and nDNA gene trees, it is essential to include other samples that can establish the 'true' haplotypes for each species; that is, conspecific individuals can be sampled from other localities where hybridization with a focal study species is deemed unlikely. These parental haplotypes can then act as reference sequences in each gene tree to compare with samples of questionable identity. Accordingly, we included individuals of *P. leptokeras* from 10 other rivers and streams, only one of which is known to contain a population of *P. anisophallos* (i.e. Japuíba River), in our mtDNA and nDNA trees, and we added individuals of *P. anisophallos* from six other rivers or streams to our mtDNA dataset and one to our nDNA datasets, including the type locality of the species, São Roque River (Lucinda 2008). By observing that most of our Parati-Mirim samples were nested in their expected (based on their morphological IDs) *P. leptokeras* or *P. anisophallos* haplotype clades (Fig. 2), we could immediately localize samples that were mis-identified using established (genitalia) and provisional (spots/no spots) morphological criteria. Given that all the other congeneric reference sequences, except for *P. enneaktinos* (see Thomaz *et al.* 2019: fig. 3), grouped with the expected mtDNA and nDNA haplotype clades (Fig. 2), we can be even more confident that the putative *P. anisophallos* and *P. leptokeras* clades reflect the true haplotypes of the species.

Low genetic diversity in *Phalloceros* species?

Another noteworthy finding from our molecular results concerns the low genetic diversity of the populations we studied. Despite having obtained mtDNA sequences from 31 individuals of *P. anisophallos* and eight of *P. leptokeras* from the Parati-Mirim River, all sampled individuals within each of these species had the same haplotype sequence (Fig. 2). A perusal of several other drainages that had multiple individuals sampled for the mtDNA gene showed the same pattern (i.e. Itamambuca, Puruba, Quiririm, Paquequer, Macacu and Perequê Açu Rivers; Fig. 2A). Only the Grande and Indaiá Rivers showed more than one distinctive haplotype sequence (Souto-Santos *et al.* 2023). These occurrences might have been attributable to gene flow that occurred when the sea level was low enough to join these adjacent rivers into one or they might represent ancestral polymorphisms that originated in the ancestral population for the two rivers. However, the low genetic diversity seen in each of these isolated populations could have arisen via bottlenecks or founder events.

Problems with identifying species of *Phalloceros*

We also found that intraspecific variation in morphology in these species can lead to incorrect species IDs, especially with immature individuals. This latter finding is of importance to researchers who study *Phalloceros* because it shows that morphology alone might be insufficient to identify individuals in systems with multiple co-occurring species of *Phalloceros*. However, our results show that standard mtDNA barcode sequences and our newly developed nDNA barcode marker provide reliable species IDs for *Phalloceros* species.

Of the 30 adult (25 female and five male) specimens in our study, only one (MNTI 14559) was misidentified using genital characters. This female individual was misidentified initially as *P. anisophallos* because it had a right-turned papilla. However, our re-examination of this specimen revealed that the papilla on this individual was aberrant; instead of pointing distally, as is the usual case for *P. anisophallos*, it was pointed in a latero-anterior direction like the normal condition for mature *P. leptokeras* females. Moreover, the papilla had a fleshy process protruding outwards from its anterior side, which is a unique characteristic of *P. leptokeras* (Souto-Santos *et al.* 2023). Although aberrantly shaped papillae have been reported for *P. harpagos*, there is negligible intraspecific variation in this trait or in the shapes of species-specific gonopodia (Lucinda 2008; Ono and Shibatta 2015; Thomaz *et al.* 2019). Thus, our study supports the practice of using genitalia to identify adult individuals of *P. leptokeras* and *P. anisophallos*.

Although our results showed that adult *Phalloceros* individuals from the Parati-Mirim River can be identified reliably as *P. anisophallos* or *P. leptokeras* based on the genital traits, we could not use these morphological features to identify the immature individuals in our sample because their genitals were either not visible (in juveniles) or they were underdeveloped (in subadults). We therefore identified these individuals provisionally using the presence or absence of dark lateral spots, following Lucinda (2008). However, substantial intraspecific variation in this trait explains the poor correlation between the presence of spots and identities of *Phalloceros* species in the Parati-Mirim River. This practice led us to misidentify four of the nine immature individuals in our sample. Most surprising of all was that 72% of our *P. anisophallos* specimens lacked spots, which contradicts Lucinda's (2008) assertion that the presence of lateral spots is a diagnostic character for this species. Although we also discovered that individuals of *P. leptokeras* from the Parati-Mirim River departed from the expected no-spot pattern (Lucinda 2008), our observations that individuals of this species can show variable spotting patterns (see Table 1) confirms the earlier findings of Souto-Santos *et al.* (2023). Thus, an important implication of our study for *Phalloceros* researchers is that immature individuals of these two species from the Parati-Mirim River can be identified only using molecular barcode sequences.

How prevalent is syntopy among species of *Phalloceros*?

Lucinda (2008: p. 156) noted that in *Phalloceros*, 'some species are sympatric or even syntopic', and then listed the pairs *P. caudimaculatus*–*P. heptaktinos* and *P. harpagos*–*P. leptokeras* as syntopic pairs of *Phalloceros*. However, Thomaz *et al.* (2019: p. 270) pointed out that *P. caudimaculatus* and *P. heptaktinos* occur in different portions of the same river basin and thus might not be syntopic. Indeed, our re-examination of Lucinda's data suggests that only the *P. harpagos*–*P. leptokeras* pair represents a valid case of syntopy in his argument. Thomaz *et al.* (2019: p. 270) performed a genus-wide analysis of species distributions for *Phalloceros* and concluded that ≥ 14 of the 22 then-known species can be found in sympatry with one or more congeners and that most of the sympatric cases represented instances of syntopy. However, our re-analysis of their data shows that Thomaz *et al.* (2019) had uncovered only four valid cases of syntopic species: *P. alessandre*–*P. pellos*, *P. malabarbai*–*P. spiloura*, *P. megapolos*–*P. pellos* and *P. megapolos*–*P. titthos* (Supporting Information, Table S3). Nonetheless, the new data we have presented here (Supporting Information, Table S3) suggest that syntopy is not only widespread in the genus, but that ≥ 14 species of *Phalloceros* occur in syntopy with a congener, thereby supporting the conjecture of Thomaz *et al.* (2019). We also found that every case of syntopy involved only two species. Moreover, when we examined these syntopic pairs in light of their likely topological placements in the *Phalloceros* species tree (Thomaz *et al.* 2019: fig. 3), all these syntopic pairs consisted of non-sister species, similar to the finding reported by Thomaz *et al.* (2019), although, again, their conclusion was based mainly on records of sympatry and not syntopy.

The independent dataset we obtained for this study also strongly corroborates the conjecture of Thomaz *et al.* (2019) concerning the prevalence of mismatched genitalia in syntopic *Phalloceros*. Indeed, all new cases of syntopy that we identified involved two coexisting species of *Phalloceros* had genitalia mismatched to some degree (Supporting Information, Table S3). This study thus confirms that syntopy is widespread in *Phalloceros* and that these species pairs seem always to consist of species with apparently mismatched genitals. However, if Lucinda's (2008) claim that *P. caudimaculatus* and *P. heptaktinos* can be found in syntopy is eventually confirmed, then this species pair would represent an intriguing exception to the syntopy/mismatched genitalia rule in *Phalloceros*. This is because the mechanical isolation hypothesis implies that co-occurring species of *Phalloceros* having 'matched' genital morphologies can interbreed with each other. The existence of such an exceptional case would thus be problematic for this hypothesis unless a future study can show that these two species are interbreeding with each other.

Do all mismatches in genitalia cause reproductive isolation?

Our results are consistent with the hypothesis that syntopic species of *Phalloceros* with mismatched genitalia are unable to hybridize with each other. However, even if this is true for the *P. anisophallos* and *P. leptokeras* populations we studied, this might not necessarily apply to other syntopic pairs of *Phalloceros*, because there are at least five different types of 'mismatched genitalia' (Table 2), which could vary in their efficacies to inhibit cross-species fertilization. Of the three genital characters that show variation at the interspecific level, divergent papillae (with or without accompanying divergences in hook number and appendix shape) characterize 11 of 17 syntopic species pairs (Table 2), hence orientation of the papillae might be a primary determinant of whether two co-occurring species of *Phalloceros* can interbreed with each other. Interestingly, the remaining six syntopic pairs showed interspecific differences in only hook number (four syntopic pairs) and hook number + shape of appendices (two pairs; Table 2). Thus, there might be other factors involved (e.g. species-specific courtship behaviours) that help to maintain the species boundaries in these six exceptional species pairs, or perhaps they interbreed with each other.

The *Phalloceros* clade is an exciting study system in which to explore the evolutionary significance of mismatched genitalia to speciation and the subsequent maintenance of species boundaries. Although we presented evidence that is consistent with the mechanical isolation hypothesis for *Phalloceros*, our findings suffer from two important limitations. First, we examined hybridization in only a small number of individuals from each population using two molecular markers. Had we sampled a larger number of individuals and/or genomic loci, we might have obtained evidence showing that *P. anisophallos* and *P. leptokeras* in the Parati-Mirim River have interbred with each other. Second, our findings, and all relevant published works on *Phalloceros*, have presented only correlational evidence in support of this hypothesis. In the future, researchers should explore the two promising avenues of population genomics and laboratory breeding experiments, which might shed much more light on the functional and evolutionary significance of mismatched genitalia in these fishes. The former approach would allow researchers to determine the levels, if any, of historical gene flow between co-occurring *Phalloceros* species that exemplify each of the observed mismatch variants listed in Table 2, and between coexisting species that have matched genitalia. In the latter approach, researchers should conduct laboratory breeding experiments between different species of *Phalloceros* to determine whether mismatched genitalia can inhibit cross-species fertilization. If the results confirm that mismatched genitalia do act as mechanical barriers to fertilization, then further experiments can be done to ascertain the relative efficacies of the different mismatch possibilities and to test the hypothesis that co-occurring species having matched genitalia are able to interbreed. Only after these studies are completed will we be able to understand fully the evolutionary significance of genital morphology variation in *Phalloceros* and its possible link to their diversification. This, in turn, might reveal the general importance of mismatched genitalia as a mechanism for reproductive isolation.

CONCLUSION

Our genetics-based findings provide correlational support for the hypothesis by Souto-Santos *et al.* (2019) and Thomaz *et al.* (2019) that mismatched genitalia between non-sister *Phalloceros* species that occur together act as mechanical isolation barriers thereby helping to maintain species boundaries. We conclude that adult female and male genitalia in *P. anisophallos* and *P. leptokeras* are reliable indicators of species ID, as was originally found by Lucinda (2008), although rare individuals might exhibit aberrant genitalia that could lead to incorrect species IDs. Our results also reinforce the finding by Souto-Santos *et al.* (2023) that the presence/absence of a dark lateral spot cannot be used to identify individuals (immatures or adults) as being *P. leptokeras*; and our findings show this to be equally true for *P. anisophallos*. Accordingly, molecular barcodes must be used to identify juvenile and immature individuals of these species wherever these species co-occur within the same body of water. Of conservation significance, we also conclude that contemporary populations of *P. anisophallos* and *P. leptokeras* have low genetic diversity.

SUPPLEMENTARY INFORMATION

Supplementary Information is available at *Zoological Journal of the Linnean Society* online.

File S1. Sequence alignment for mitochondrial DNA *COI* gene sequences in nexus format.

File S2. Sequence alignment for nuclear DNA *RAG1* gene sequences in nexus format.

Table S1. Specimens, localities, voucher numbers and GenBank numbers. 'N/A' means not available. Note that MNRJ7028 was previously identified as *Phalloceros tupinamba* (see Jennings *et al.* 2019) but is now *Phalloceros leptokeras* (see Souto-Santos *et al.* 2023). Note that species identifications in this table are based on morphological criteria (see Materials and Methods). Abbreviations: MNLM, DNA Extract Collection, Laboratório de Pesquisa em Diversidade Molecular, Museu Nacional, Universidade Federal do Rio de Janeiro (UFRJ); MNRJ, Ichthyological Collection, Museu Nacional, UFRJ; MNTI, Fish Tissue Collection, Museu Nacional, UFRJ.

Table S2. Identification of specimens sampled from the Parati-Mirim River, Rio de Janeiro state as belonging to *Phalloceros anisophallos* or *Phalloceros leptokeras*. 'N/A' means not applicable. Abbreviations: MNLM, DNA Extract Collection, Laboratório de Pesquisa em Diversidade Molecular, Museu Nacional, Universidade Federal do Rio de Janeiro (UFRJ); MNRJ, Ichthyological Collection, Museu Nacional, UFRJ; MNTI, Fish Tissue Collection, Museu Nacional, UFRJ.

Table S3. Voucher specimen-based records of syntopy in the genus *Phalloceros*. Original geographical coordinates were obtained from GPS at each collection site, except for localities with an asterisk (*), which we estimated from topographic maps. Decimal coordinates for records from the paper by Thomaz *et al.* (2019) are converted into degrees, minutes and seconds. Abbreviations: MNRJ, Ichthyological Collection, Museu Nacional, Universidade Federal do Rio de Janeiro; UFRGS, Ichthyological Collection at the Universidade Federal do Rio Grande do Sul. Note that *P. maldonadoi* is a newly described species of *Phalloceros* (see Souto-Santos *et al.* 2023).

ACKNOWLEDGEMENTS

We are grateful to the Associate Editor, Paulo Lucinda, and an anonymous reviewer for providing us with constructive comments that have greatly improved this manuscript. This research was supported by a foreign visiting researcher fellowship from the Coordenação de Aperfeiçoamaento de Pessoal de Nível Superior (CAPES) to W.B.J. (number 8887.571413/2020-00) and a CAPES grant to E.Z. (number 88887.311746/2018-00) and to P.A.B. (number 88882.156885/2016-01), grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) to P.A.B. (numbers 312801/2017-3 and 423526/2018-9), CNPq doctoral fellowship grants to I.C.A.S.-S. (number 140559/2021-3), Fundação de Amparo à Pesquisa no Estado do Rio de Janeiro (FAPERJ) grants to P.A.B. (numbers E406 26/200.063/2019 and E-26/203.106/2019) and E.Z. (numbers E-26/201.286/2021, E-26/211.078/2019 and E-26/203.213/2017), and a Universidade do Estado do Rio de Janeiro faculty Prociência grant to E.Z. Gustavo Andrés Ferraro provided technical assistance for laboratory processing of part of the samples.

DATA AVAILABILITY

All DNA sequences and associated data are available in the Barcode of Life Data Systems (BOLD Systems; http://boldsystems.org/) and GenBank under process IDs and accession numbers listed in the Supporting Information (Table S1). Multiple DNA sequence alignments used in phylogenetic analyses are available as Supporting Information documents. All morphological data used in this study are available in the Supporting Information (Table S2).

CONFLICTS OF INTEREST

The authors declare they have no conflict of interest.

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