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Uncertainties and risks in delimiting species of *Cambeva* (Siluriformes: Trichomycteridae) with single-locus methods and geographically restricted data

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Cambeva contains species with complex taxonomy or poorly delimitated in terms of morphology and geopraphic distribution. We conducted an extensive review of *Cambeva* populations from coastal drainages of Southern to Southeastern Brazil to evaluate species geographic limits with an integrative analysis including morphological and molecular data (COI). We test if two single-locus methods, Bayesian Poisson Tree Processes (bPTP) and Generalized Mixed Yule Coalescent (GMYC), are efficient to delimit species boundaries in *Cambeva* by the comparison with the diagnosable morphological units. Using GMYC, we also evaluated the combination of tree and molecular clock priors to reconstruct the input phylogeny and assessed how well the implemented model fitted our empirical data. Eleven species were identified using a morphological diagnosability criterion: *Cambeva balios*, *C. barbosae*, *C. botuvera*, *C. cubataonis*, *C. davisi*, *C. guaraquessaba*, *C. iheringi*, *C. tupinamba*, and *C. zonata* and two treated as undescribed species. In contrast with previous knowledge, many of them have wider distribution and high intraspecific variation. Species delimitation based on single-locus demonstrated incongruences between the methods and strongly differed from the morphological delimitation. These disagreements and the violation of the GMYC model suggest that a single-locus data is insufficient to delimit *Cambeva* species and the failure may be attributable to events of mitochondrial introgression and incomplete lineage sorting.

Keywords: Color pattern, GMYC, Integrative taxonomy, Species delimitation, Trichomycterinae.

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Cambeva contém espécies com taxonomia complexa ou mal delimitadas em termos morfológicos e de distribuição geográfica. Realizamos uma extensa revisão de populações de *Cambeva* das drenagens costeiras do Sul ao Sudeste do Brasil para avaliar os limites das espécies com uma análise integrativa incluindo dados morfológicos e moleculares (COI). Testamos se dois métodos de locus único, Implementação Bayesiana dos Processos da Árvore de Poisson (bPTP) e Coalescente de Yule Misto Generalizado (GMYC), são eficientes para delimitar os limites das espécies em *Cambeva* pela comparação com as unidades morfológicas diagnosticáveis. Usando o GMYC, também avaliamos a combinação de árvores e relógios moleculares para reconstruir a filogenia e avaliamos o quão bem o modelo implementado se ajustava aos nossos dados empíricos. Foram identificadas 11 espécies usando o critério morfológico: *Cambeva balios*, *C. barbosae*, *C. botuvera*, *C. cubataonis*, *C. davisi*, *C. guaraquessaba*, *C. iheringi*, *C. tupinamba* e *C. zonata* e duas tratadas como espécies não-descritas. Em contraste com o conhecimento prévio, muitas delas têm distribuição mais ampla e alta variação intraespecífica. A delimitação das espécies baseada em locus único demonstrou incongruências entre os métodos e diferiu fortemente da delimitação morfológica. Essas discordâncias e a violação do modelo GMYC sugerem que os dados de locus único são insuficientes para delimitar as espécies de *Cambeva* e a falha pode ser atribuída a eventos de introgressão mitocondrial e sorteio incompleto da linhagem.

Palavras-chave: Delimitação de espécie, GMYC, Padrão de coloração, Taxonomia integrativa, Trichomycterinae.

INTRODUCTION

The family Trichomycteridae contains 371 species (Fricke *et al*., 2022) distributed exclusively in the Neotropical region, the second richest Siluriformes family. The subfamily Trichomycterinae includes 270 species classified in nine genera, *Bullockia* Arratia, Chang, Menu-Marque & Rojas, 1978, *Cambeva* Katz, Barbosa, Mattos & Costa, 2018, *Eremophilus* Humboldt, 1805, *Hatcheria* Eigenmann, 1909, *Rhizosomichthys* Miles, 1943, *Scleronema* Eigenmann, 1917, *Trichomycterus* Valenciennes, 1832, *Ituglanis* Costa & Bockmann, 1993, and *Silvinichthys* Arratia, 1998; which represent more than two-thirds of the family diversity (Fricke *et al*., 2022). In terms of phylogenetic studies, the family has been investigated using anatomical systems [*e.g*., osteology (de Pinna, 1998) and musculature (Datovo, Bockmann, 2010)], and molecular data as the multi-locus approaches (Ochoa *et al*., 2017a; Henschel *et al*., 2018; Katz *et al*., 2018; Fernández *et al*., 2021) and with ultraconserved elements (Ochoa *et al*., 2020). As a result, there were significant advances in phylogeny and classification, such as establishing the monophyletic status of Trichomycterinae and rearrangements of its internal clades.

One of these clades units within Trichomycterinae was firstly namely "clade D4" by Ochoa *et al*. (2017a) and consists of *Scleronema* plus a subset of species of *Trichomycterus*, a long-standing "waste-basket" genus of Trichomycteridae (de Pinna, 1998). Posteriorly, Katz *et al*. (2018) proposed the genus *Cambeva* to allocate those species that formed a sister clade of *Scleronema* and currently contains 46 valid species (Fricke *et al*., 2022).

One of the puzzles to delimit trichomycterine species is associated with their variable intraspecific morphology. Color pattern, for example, is often used to diagnose Neotropical freshwater fishes but has been demonstrated to be labile for species of *Cambeva* and *Trichomycterus* due to aspects as ontogeny, geographic variation, or habitat specificity (see Arratia *et al*., 1978; Silva *et al*., 2010; Ferrer, Malabarba, 2013; Nascimento *et al*., 2017; Donin *et al*., 2020; Pereira *et al*., 2021). Besides that, the morphological variation of trichomycterine species is repeatedly poorly accessed, and some descriptions are conducted based solely on specimens from type localities (Costa, 1992; Triques, Vono, 2004; Wosiacki, Oyakawa, 2005; Barbosa, Costa, 2010; Costa *et al*., 2020a,b). Consequently, some of these species' hypotheses need reevaluation after a careful examination of new samples, including ontogenetic series and broad geographic areas (Reis, de Pinna, 2019; DoNascimiento, Prada-Pedreros, 2020; Donin *et al*., 2020; Lima *et al*., 2021).

Single-locus sequence-based species delimitations have been increasingly used to resolve species limits in the diverse Neotropical fish fauna (Serrano *et al*., 2019; Agudelo-Zamora *et al*., 2020; Delapieve *et al*., 2020; Mateussi *et al*., 2020; Lima *et al*., 2021). In this regard, some methods using DNA sequences have an exploratory capacity, do not require previous information on species assignment, and are helpful in groups with uncertain and problematic alpha-taxonomy (Talavera *et al*., 2013; Luo *et al*., 2018). Currently, the most frequent tree-based methods applied to species delimitation are the Generalized Mixed Yule Coalescent (GMYC) and the Bayesian Poisson Tree Processes (bPTP), which either use branch lengths to identify branching rate transition points or the number of substitutions (Fujisawa, Barraclough, 2013; Zhang *et al*., 2013).

Although these methods have been recognized as a valuable tool to delimit species, critics regarding its methodological deficiencies, such as over splitting, limited information on single-locus analysis, and accuracy concerning different priors on inputted phylogenetic reconstructions (Talavera *et al*., 2013; da Cruz, Weksler, 2018). Additionally, their accuracy depends on a sampling of multiple individuals within each species, small effective population sizes, and the absence of population genetic structure within species (Fujisawa, Barraclough, 2013; Luo *et al*., 2018; Fonseca *et al*., 2021).

In this paper, an extensive review of *Cambeva* populations from coastal drainages of Southern to Southeastern Brazil was used to evaluate species limits in an integrative analysis with morphological and molecular data. The main question is whether single-locus methods, specifically GMYC and bPTP, are efficient to delimit species boundaries in the genus *Cambeva*. Additionally, we describe intraspecific variations and updated geographic distributions for the species of *Cambeva* in the study area and discuss their relevance facing previous taxonomic papers.

MATERIAL AND METHODS

Study region and taxa sources. The study area ranges from Tramandaí River basin to small rivers in the São Paulo State, representing the known southern and northern limits, respectivaly of *Cambeva* in Brazilian coastal drainages (Fig. 1). We conducted a population level analysis (per basin) in the following drainages (south to north): Tramandaí River, Mampituba River, Araranguá River, Tubarão River, Cubatão Sul

River, Biguaçu River, Tijucas River, Santa Catarina Island streams, Itapocu River, Cubatão Norte River, tributaries to Babitonga, Guaratuba and Paranaguá bays, Ribeira de Iguape River, and small coastal rivers of São Paulo State.

All fishes were anesthetized with a Eugenol solution to take pictures and extract tissue samples from muscle or fin clips. Photographs of live specimens were taken by TPC using the phototank immersion method proposed by Sabaj Pérez (2009). Posteriorly, the fishes were euthanized with a concentrated Eugenol solution (Lucena *et al*., 2013) and fixed in 10% formaldehyde. Currently, the specimens and the tissue samples are preserved in 70% and 96% ethanol, respectively, in the fish collection of the Departamento de Zoologia, Universidade Federal do Rio Grande do Sul, Porto Alegre (UFRGS). Additional specimens and tissue samples used in the study are listed in the Tab. **S1** and material examined.

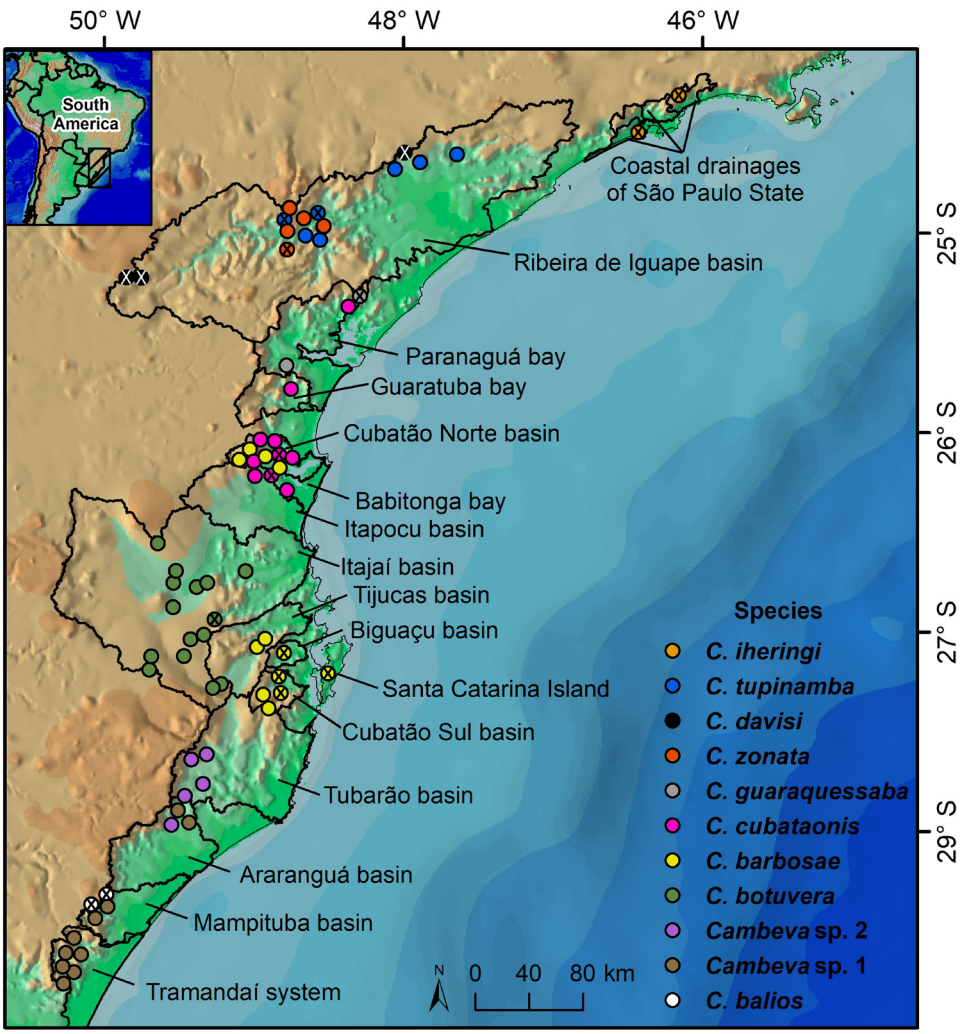

FIGURE 1 | Geographical distribution of *Cambeva* species in the coastal drainages of Southern and Southeastern Brazil (crossed circles indicate their past known distribution). Some dots represent more than one locality.

Morphological data and morphospecies assignment. Primary species (or morphospecies) assignment was done based on a combination of morphological characters, typically used in the alpha-taxonomy of *Cambeva* (*e.g.*, color pattern, morphometric, fin-ray counts, osteology, and pores of the laterosensory system), which enabled us to diagnose species within the study area. These characters were searched in the original descriptions (Ferrer, Malabarba, 2013; Costa *et al.*, 2021; Bizerril, 1994; Haseman, 1911; Wosiacki, 2005; Eigenmann, 1917, 1918; Wosiacki, Oyakawa, 2005), newly taxonomic works (Wosiacki, 2005; Malabarba *et al.*, 2013; Nascimento *et al.* 2017; Donin *et al.*, 2020) or currently proposed. The following measurements were taken point to point with a digital caliper (precision 0.1 mm): (1) standard length, (2) head length, (3) head width, (4) pre-dorsal length, (5) pre-pelvic length, (6) pre-anal length, (7) scapular-girdle width, (8) trunk length, (9) pectoral-fin length, (10) pelvic-fin length, (11) distance between pelvic-fin base and anus, (12) caudal-peduncle length, (13) caudal-peduncle depth, (14) body depth, (15) dorsal-fin base length, (16) anal-fin base length, (17) snout length, (18) interorbital distance, and (19) eye diameter (Fig. 2); maxillary, nasal, and rictal barbels lengths (Tchernavin, 1944); mouth width and supraorbital pore s6 distance (Costa, 1992); and interopercular patch length (Ferrer *et al.*, 2015). Principal component analysis (PCA) was applied to check the overall morphometric variation among populations and species with similar morphology and genetics, using measurements treated for size using a log-ratio transformation (Aitchinson, 1982). Osteological information was obtained from cleared and counterstained specimens (cs) according to procedures described by Taylor, Van Dyke (1985). Vertebrae counts exclude those of the Weberian complex, and the compound

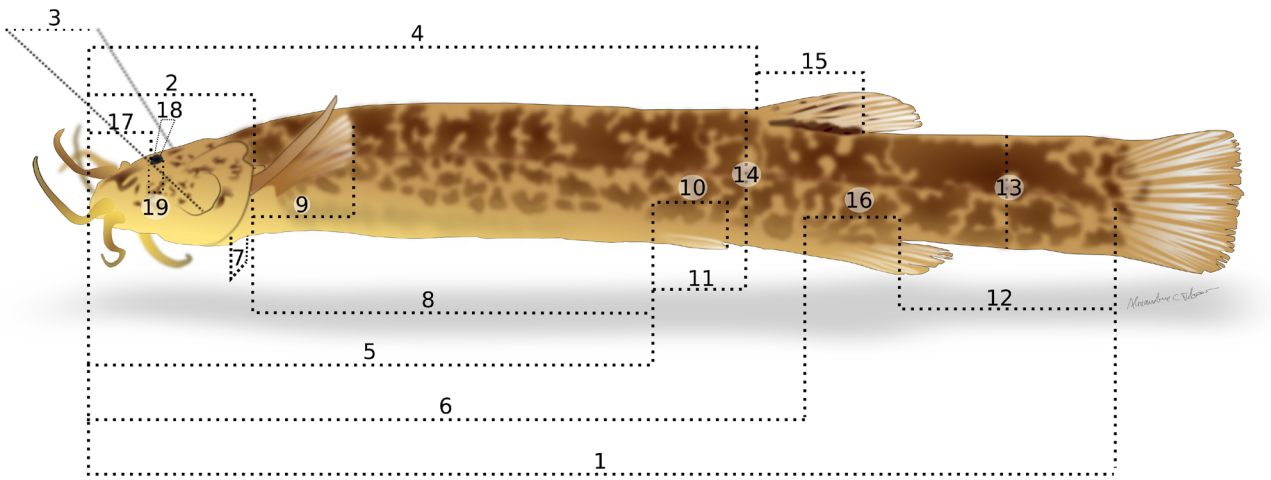

FIGURE 2 | Schematic drawing of morphometric measurements applied to *Cambeva* species: (1) standard length, (2) head length, (3) head width, (4) predorsal length, (5) prepelvic length, (6) pre-anal length, (7) scapular girdle width, (8) trunk length, (9) pectoral-fin length, (10) pelvic-fin length, (11) distance between pelvic-fin base and anus, (12) caudal peduncle length, (13) caudal peduncle depth, (14) body depth, (15) length of dorsal-fin base, (16) length of anal-fin base, (17) snout length, (18) interorbital distance, and (19) eye diameter. Illustration made by Alexandre Ribeiro.

caudal centrum (PU1+ U1) was counted as a single element. Counts of unbranched and branched rays of the pectoral fin are represented by upper case Roman numeral and Arabic numerals, respectively. Nomenclature of the laterosensory canals and associated pores follows Rizzato, Bichuette (2016).

Molecular data and analyses. Genetic sequences used in the analyses are those cited by Donin *et al*. (2020; reference number: 7) in addition to others available in GenBank from coastal drainages and neighboring continental basins associated with the following papers: Silva *et al*. (2010; 1), Pereira *et al*. (2013; 2), Nascimento *et al*. (2017; 3), Ochoa *et al*. (2017a,b; 4), Katz *et al*. (2018; 5), and Morais-Silva *et al*. (2018; 6) (Tab. **S1**). In total, 170 sequences and 23 terminal taxa were used (Tab. **S1**). We adopted the taxonomic identification provided by these authors citing their authorship in the text and making their reference in the phylogenetic trees through the reference numbers mentioned above. The sequence identified as *Trichomycterus paolence* Eigenmann, 1917 (currently *Cambeva*; GenBank accession HM376398) from Pereira *et al*. (2013) do not align with any sequence of *Cambeva* and was not included in the analyses. The DNA extractions, PCR, sequences editing, and alignment followed Donin *et al*. (2020). We applied two different methods of species delimitation using fragments of the mitochondrial DNA cytochrome oxidase c subunit I (COI) to examine congruence between these and the delimitated morphospecies: Generalized Mixed Yule Coalescent (GMYC; Fujisawa, Barraclough, 2013; Pons *et al*., 2006) and Bayesian Poisson tree processes (bPTP; Zhang *et al*., 2013). The methods differ in that GMYC uses branch lengths (timed divergences) to identify when divergence times more closely resemble coaslescence events on a time-calibrated ultrametric tree, while in PTP the method model speciation by using the number of substitutions, and a ultrametric tree is not required (Zhang *et al*., 2013).

The COI alignment was partitioned by codon position to determine the best models and partition schemes of molecular evolution in PartitionFinder v.1.1.1 (Lanfear *et al*., 2012) under the Bayesian information criterion (BIC). To conduct the GMYC analysis, we assess the impact of four different combinations of priors [clock models (strict and relaxed lognormal) and tree priors (yule and a coalescent prior of constant population size)] on the delimitation of species (defined here as combination 1, 2, 3 and 4). Using these combinations of distinct priors (clock model and tree prior) we estimated an ultrametric tree, because the model requires an ultrametric tree as input (Fujisawa, Barraclough, 2013), in BEAST v.2.5.0 (Drummond *et al*., 2012), programmed to run for 40 million generations (MCMC), sampling every 4×10^3 generations. The convergence was assessed by assuring that all parameters reached stationarity and effective sample sizes (*i.e*., ESS > 200) using Tracer v.1.7.1 (Rambaut *et al*., 2018). The first 4 million generations (10%) were discarded as burn-in of 10%, and the remaining trees were used to summarize the results of the Bayesian analysis, using the Maximum Clade Credibility Tree (MCC) in TreeAnnotator BEAST v.1.7 (Rambaut, Drummond, 2013). The MCC was checked in FigTree v1.4.3 and used as an input file (Newick format) for the GMYC analyses performed in the GMYC server (https://species.h-its.org/gmyc/) under a single threshold method. We also assessed the statistical fit of the GMYC model using the parametric bootstrap (100 replications) approach of the R package P2C2M. GMYC (Fonseca *et al*., 2021) for all the four inputted phylogenetic trees that used a distinct combination of priors in BEAST. P2C2M.GMYC calculates the number of

species clusters for the empirical dataset then uses the number of inferred species to simulate a Yule tree and coalescent genealogies for each terminal lineage using the number of individuals inferred to be in each species cluster. Then simulate sequences and a UPGMA tree for each tree simulated under the correct model and compare the empirical and simulated number of species after replicates. This procedure is suggested to be included in the analytical pipeline of GMYC analyses (Fonseca *et al*., 2021).

In the Bayesian Poisson tree processes (bPTP), we generated a non-ultrametric best maximum likelihood (ML) tree implemented in IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos *et al*., 2016) under an Ultrafast Bootstrap analysis (Hoang *et al*., 2018) and the remaining of the parameters with default values. The best model for nucleotide substitution was estimated under a Bayesian criterion (BIC) in IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/). The bPTP analysis was performed in the bPTP server (https://species.h-its.org/ptp/) under default values.

Collection abbreviations. CPUFMT, Coleção de Peixes da Universidade Federal do Mato Grosso, Cuiabá; DZSJRP, Coleção de Peixes da Universidade Estadual Paulista Júlio de Mesquita Filho, São José do Rio Preto; FMNH, Division of Fishes, Department of Zoology, Field Museum of Natural History, Chicago; MCN, Museu de Ciências Naturais (ex-Fundação Zoobotânica do Rio Grande do Sul), SEMA-RS, Porto Alegre; MCP, Museu de Ciências e Tecnologia da Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre; MNRJ, Museu Nacional, Rio de Janeiro; MPEG, Museu Paraense Emílio Goeldi, Zoologia, Laboratório de Ictiologia, Belém; MZUEL, Museu de Zoologia da Universidade Estadual de Londrina, Londrina; MZUSP, Museu de Zoologia da Universidade de São Paulo, São Paulo; NUP, Coleção Ictiológica do Nupélia, Universidade Estadual de Maringá, Maringá; UNICTIO, Laboratório de Ictiologia, Universidade do Vale do Rio dos Sinos, São Leopoldo.

RESULTS

Morphospecies identity and geographic distribution. We recognized 11 morphospecies of *Cambeva* across the study area (Fig. 1; Tab. 1). Nine of them can be associated with valid species names: *Cambeva balios* (Ferrer & Malabarba, 2013), *C. barbosae* Costa, Feltrin & Katz, 2021, *C. botuvera* Costa, Feltrin & Katz, 2021, *C. cubataonis* (Bizerril, 1994), *C. davisi* (Haseman, 1911), *C. guaraquessaba* (Wosiacki, 2005), *C. iheringi* (Eigenmann, 1917), *C. tupinamba* (Wosiacki & Oyakawa, 2005), and *C. zonata* (Eigenmann, 1918), and two are treated as undescribed species (*Cambeva* sp. 1 and *Cambeva* sp. 2).

In the study area, *C. balios* (Fig. 3A) occurs exclusively in the headwaters of the Mampituba River basin. *Cambeva balios* is distinguished from its congeners in the coastal basins, except *C. botuvera*, by the coloration with two distinct layers in adult specimens composed of large rounded black blotches in the inner layer and small spots in the outer layer over a pale-yellow background (*vs*. body with mottled pattern of blotches not rounded, with spots or rounded blotches restricted to form a mid-lateral line, or with small circular blotches not coalescent). It differs from *C. botuvera* by having 10–13 ventral procurrent caudal-fin rays (*vs*. 14–15).

FIGURE 3 | A. *Cambeva balios* from Mampituba River basin, UFRGS 16295, 63.8 mm SL. **B.** *Cambeva davisi* from Ribeira de Iguape River basin, MZUEL 17202, 72.8 mm SL, fixed in alcohol. **C.** *Cambeva iheringi* from Piagui River, coastal drainage of São Paulo State, MNRJ 24008, 75.6 mm SL.

TABLE 1 | Primary species (or morphospecies) recognized along the study area and their diagnostic morphological characters typically used in the alpha-taxonomy of *Cambeva* from coastal basins of South and Southeast Brazil.

Morphospecies/ characters	Pectoral-fin rays: modal number	Dorsal procurrent caudal-fin rays: number	Ventral procurrent caudal-fin rays: number	Branchiostegal rays: number	Vertebrae: number	Ribs: number	First pterygiophore of dorsal fin position: anterior to neural spine of vertebra	First pterygiophore of anal fin position: anterior to hemal spine of vertebra	Antorbital segment of the infraorbital canal (pores 'i1', 'i3')	Presumably adults body: overall coloration
Cambeva balios	I,6	14–16	12	9	39–40	12–13	20–21	22–23	absent	Two distinct layers composed of large rounded black blotches in the inner layer and small spots in the outer layer over a pale yellow background
Cambeva barbosae	I,7	17–22	10–12	7–8	36–37	11–12	16–18	21–22	absent	Mottled pattern composed of irregular and coalescent inconspicuous black or with sparse black blotches varying in, eventually rounded, over a pale yellow background
Cambeva botuvera	I,6	17–19	14–15	8	39–40	12–14	20–21	23	absent	Two distinct layers composed of large ellipsoid black blotches, sometimes coalescent and forming a mid-lateral stripe, vermicular black marks, or yet inconspicuous black spotted in the inner layer; and small spots in the outer layer over a pale yellow background
Cambeva cubataonis	I,6	17–21	12–13	9	38–40	12–15	18–20	22–24	usually present	Mottled pattern composed of coalescent black blotches sometimes forming saddle marks dorsally, over a pale yellow background or almost completely black with thin yellowish areas
Cambeva davisi	I,6	17	11	9–10	37–38	13–15	19	22–23	absent	Mottled pattern composed of coalescent and irregular black marks over a pale yellow background
Cambeva guaraquessaba	I,7	15	8	8	37	14–15	18	22	present	Light brown background with a mid-lateral line composed of black spots with about the eye size
Cambeva iheringi	I,7	18	8	7–8	35	11	15–16	21–22	present	Numerous black spots or sparse rounded black blotches, sometimes forming a mid-lateral line, over a grayish or a pale yellow background
Cambeva tupinamba	I,7	15–16	10	7–8	39–40	14–15	19–20	23–25	present	Brown background with a mid-lateral black stripe along the trunk and sparse black spots
Cambeva zonata	I,6	13–15	9–12	8–9	37	13	18–19	21	absent	Mottled pattern composed of coalescent black marks, sometimes forming a mid-lateral stripe over a pale yellow background, and never with saddle marks dorsally
***Cambeva* sp. 1**	I,5	14–17	10–12	8–9	36–39	12–14	17–20	20–23	absent	Mottled pattern composed of coalescent and irregular black marks over a pale yellow background
***Cambeva* sp. 2**	I,6	17–21	12–14	7–8	38–40	14–15	19	22–23	absent	Pale yellow background with small circular black blotches not coalescent

Cambeva barbosae (Fig. 4) is distributed in the Cubatão Sul, Biguaçu, Tijucas, Itapocu, Cubatão Norte river basins and in the Santa Catarina Island. *Cambeva barbosae* is distinguished from its congeners by the combination of a high number of pectoral-fin rays modally I,7 (*vs*. I,5 or I,6), the antorbital segment of the laterosensory system (pores i1 and i3) absent (*vs*. present), and by the coloration with a mottled pattern composed of irregular and coalescent inconspicuous black marks (Figs. 4A, B) or with sparse black blotches varying in size (Figs. 4C, D), eventually rounded (Fig. 4E) (*vs*. body with large rounded or ellipsoid black blotches; with spots or rounded blotches forming a mid-lateral line, with small circular blotches not coalescent).

FIGURE 4 | Polymorphic color pattern within of *Cambeva barbosae* ranging from a mottled color pattern in individuals from (**A**) Santa Catarina Island, UFRGS 23183, 80.1 mm SL, and (**B**) Biguaçu River basin, UFRGS 22907, 61.3 mm SL; to a blotched color pattern with dark marks varying in shape and size from Biguaçu River basin, (**C, D**) UFRGS 22932, 58.7mm SL and 51.0 mm SL, respectively, and (**E**) UFRGS 20937, 30.0 mm SL.

Cambeva botuvera (Fig. 5) is widely distributed in the Itajaí River basin being distinguished from other congeners in the coastal area, except *C. balios*, by the coloration with two distinct layers in adult specimens composed of large ellipsoid black blotches (Figs. 5A, F), sometimes coalescent and forming a mid-lateral stripe (Figs. 5B, D), vermicular black marks (Fig. 5E), or yet inconspicuous black-spotted (Fig. 5C) in the inner layer; and small spots in the outer layer over a pale yellow background (*vs*. body with mottled pattern of blotches not ellipsoid, with spots or rounded blotches restricted to form a mid-lateral line, or with small circular blotches not coalescent). It differs from *C. balios* by having 14–15 ventral procurrent caudal-fin rays (*vs*. 10–13).

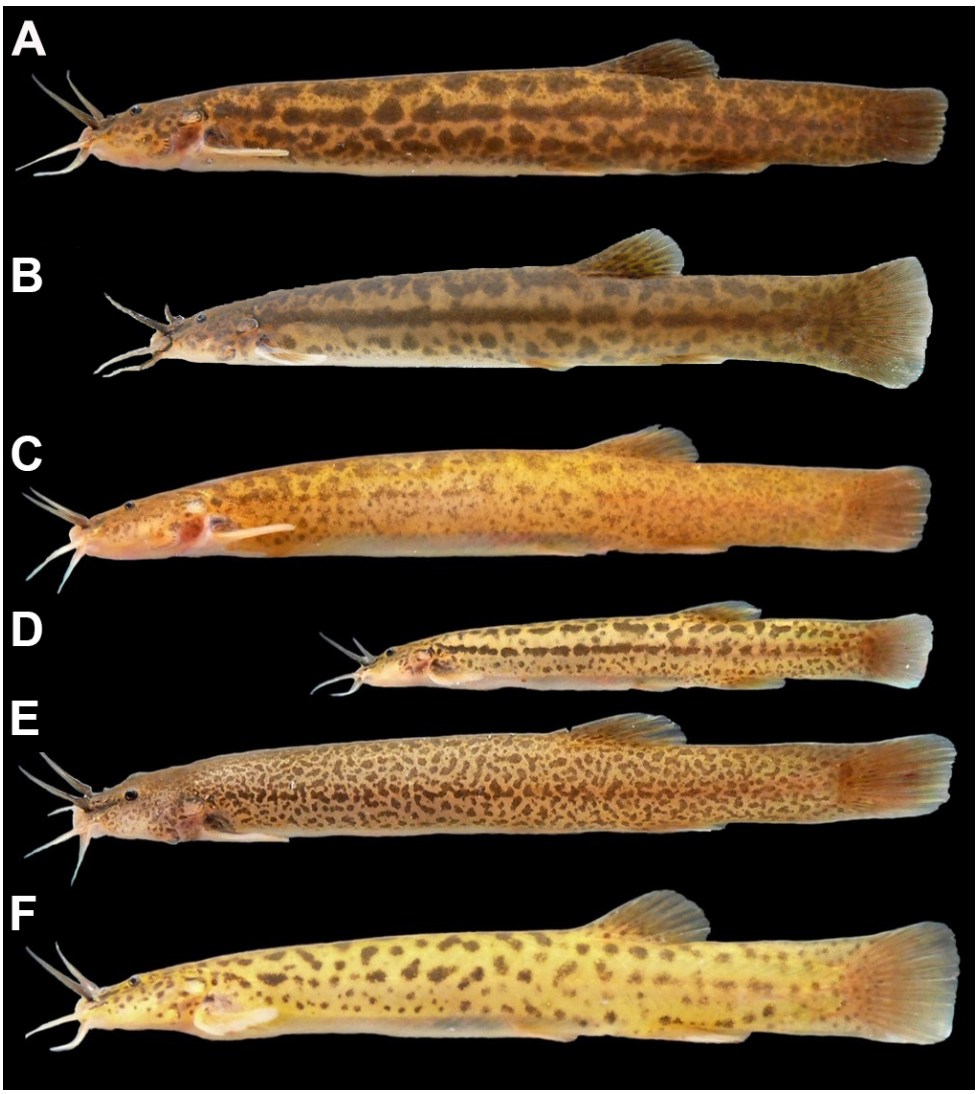

FIGURE 5 | Variation on the coloration in vivo and ontogenetic series of *Cambeva botuvera* from (**A, C–F**) Itajaí-Açu River, UFRGS 23182, 47.3 mm SL, 64.8 mm SL, 68.1 mm SL, 81.1 mm SL, 95.6 mm SL, respectively; and (**B**) from Itajaí-Mirim River, UFRGS 24558, 67.1 mm SL, showing the color pattern with two layers in the skin composed of large and small round black blotches in the inner and outer layer, respectively.

Cambeva cubataonis (Fig. 6) is distributed in the Itapocú River, Cubatão Norte River, and tributaries of Babitonga, Guaratuba and Paranaguá bays. *Cambeva cubataonis* is distinguished by the antorbital segment of the laterosensory system (pores i1 and i3) usually present (variable only in a few individuals from Babitonga and Guaratuba bays) (*vs.* always absent), the number of pectoral-fin rays modally I,6 (*vs.* I,5 or I,7), and coloration with a mottled pattern composed of coalescent black blotches sometimes forming saddles dorsally, over a pale-yellow background (Figs. 6A, B) or almost entirely black with thin yellowish areas (*vs.* body with mottled pattern not forming saddles dorsally; with large rounded or ellipsoid black blotches; with spots or rounded blotches forming a mid-lateral line, or with small circular blotches not coalescent) (Figs. 6C, D).

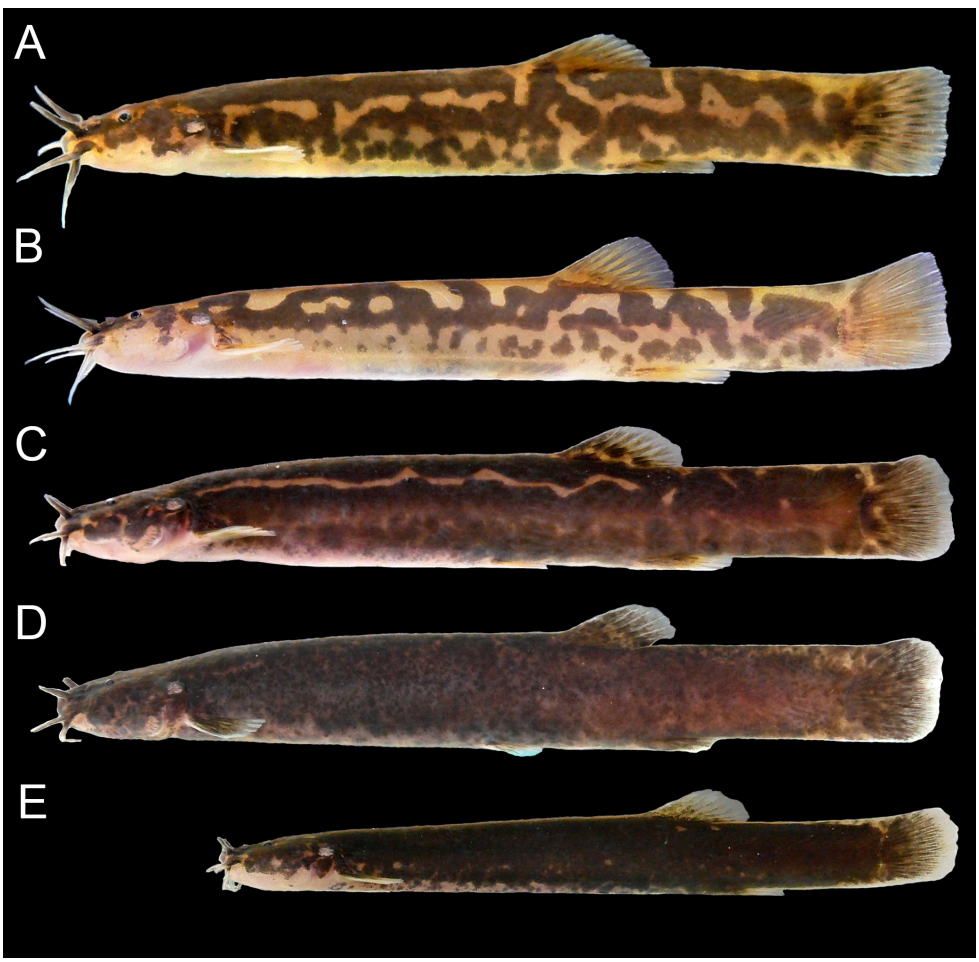

FIGURE 6 | Variation on the coloration in *Cambeva cubataonis*, showing a mottled color pattern composed by blotches variable in shape and forming marked saddles dorsally, (**C–E**) specimens with large dark areas merged giving an overall dark color pattern. Specimens from tributaries of Guaratuba Bay (**A**) UFRGS 24549, 53.1 mm SL; (**B**) UFRGS 24556, 60.3 mm SL; and from tributaries to Babitonga Bay (**C–E**) UFRGS 24552, 77.2 mm SL, 85.7 mm SL, and 61.6 mm SL, respectively.

Cambeva davisi (Fig. 3B) is restricted to the headwaters of the Ribeira de Iguape River basin being distinguished from its congeners by its mottled color pattern composed of coalescent and irregular black marks (*vs*. mottled pattern forming saddles dorsally; body with large rounded or ellipsoid black blotches; with spots or rounded blotches forming a mid-lateral line, with small circular blotches not coalescent) the first anal-fin pterygiophore placed anteriorly to the 22nd–23rd hemal spines of the vertebral column (*vs*. anteriorly to the 20–25th), and the antorbital segment of the laterosensory system (pores i1 and i3) absent (*vs*. present).

Cambeva iheringi (Fig. 3C) is known from coastal drainages along the Serra do Mar escarpment in the São Paulo State. *Cambeva iheringi* is distinguished from its congeners by the low number of vertebrae (35 *vs*. 36–40) and its coloration composed of numerous black spots or sparse rounded black blotches, sometimes concentrated along de mid-lateral line of the body, over a pale-yellow or a grayish background (*vs*. body with mottled pattern of blotches).

Cambeva guaraquessaba (Figs. 7A, B) occurs exclusively in the tributaries to the Paranaguá Bay, being distinguished from its congeners by the color pattern with a mid-lateral line composed of black spots with about the eye size (Figs. 8A, B) (*vs*. body with blotches or spots not forming a mid-lateral stripe), 37 vertebrae (*vs*. 35–40), and the first dorsal- and anal-fin pterygiophores placed anteriorly to the 18th neural spine and 22nd hemal spine of the vertebral column, respectively (*vs*. anteriorly to the 16th–21st and 20–25th, respectively).

FIGURE 7 | Coloration pattern of *Cambeva* species. (**A, B**). *Cambeva guaraquessaba*, UFRGS 24554, 37.0 and 36.9 mm SL, respectively, tributary of Guaraqueçaba River basin, Paranaguá Bay. **C–D**. *Cambeva tupinamba*, UFRGS 24550, 63.0 mm SL and 39.4 mm SL, Betari River, Ribeira de Iguape River basin.

Cambeva tupinamba (Figs. 7C, D) occurs exclusively in the Ribeira de Iguape River basin being distinguished from its congeners by the color pattern with a series of spots forming anteriorly (to the anal-fin origin) a mid-lateral black stripe along the trunk (Figs. 7C, D) (*vs*. body with blotches or spots not forming a mid-lateral stripe), 39 to 40 vertebrae (*vs*. 35–40), the first dorsal- and anal-fin pterygiophores placed anteriorly to the 19th–20th neural spine and 23th–25th hemal spine of the vertebral column, respectively (*vs*. anteriorly to the 15th–21st; 20–24th, respectively).

Cambeva zonata (Fig. 8) is restricted to the Ribeira de Iguape River basin along the Betari River and Iporanga River sub-basins. *Cambeva zonata* is distinguished by its mottled color pattern composed of coalescent black marks, sometimes forming a mid-lateral stripe in juveniles, but with no clear saddled pattern (*vs.* body with mottled pattern forming saddles dorsally; with large rounded or ellipsoid black blotches; with spots or rounded blotches forming a mid-lateral line, or with small circular blotches not coalescent); the first anal-fin pterygiophore placed anteriorly to the 21th hemal spine of the vertebral column (*vs*. anteriorly to the 22nd–24th), and the antorbital segment of the laterosensory system (pores i1 and i3) absent (*vs*. present).

FIGURE 8 | Coloration pattern of *Cambeva zonata*, (**A–C**) UFRGS 24538, (**A**) 51.1 mm SL, (**B**) 48.4 mm SL, Betari River, Ribeira de Iguape River basin, and (**C**) 41.4 mm SL.

In the coastal region, *Cambeva* sp. 1 (Fig. 9) is the southernmost morphospecies inhabiting the Tramandaí River system (specifically the Maquiné and Três Forquilhas river basin), Mampituba, and Araranguá river basins. *Cambeva* sp. 1 is distinguished from its congeners by the lower number of pectoral-fin rays (modally I,5 *vs*. I,6 or I,7).

Cambeva sp. 2 (Fig. 10) is distributed in the Araranguá and Tubarão river basins and differs from its congeners by a color pattern composed of circular black blotches, about eye size or slightly larger, that are not coalescent (body with mottled pattern of coalescent blotches, with spots or rounded blotches restricted to form a mid-lateral line). Additionally, *Cambeva* sp. 2 is distinguished from *C. balios* and *C. botuvera* (both eventually have a similar color pattern in juveniles) by the higher number of dorsal procurrent caudal-fin rays (17–21 *vs*. 10–16), and the first dorsal-fin pterygiophore placed anteriorly to the 19th neural spine of the vertebral column (*vs*. anteriorly to the 20th–21st).

FIGURE 9 | ndividuals representing populations of *Cambeva* sp. 1 from distinct river drainages. (**A**) Maquiné River basin, UFRGS 22211, 58.6 mm SL, (**B**) Mampituba River basin, MCP 23623, 55.7 mm SL; and (**C**, **D**) Araranguá River basin, UFRGS 22962, 45.2 and 59.8 mm SL, respectively.

FIGURE 10 | Variation on the coloration of *Cambeva* sp. 2 from (**A–D**) Tubarão River, UFRGS 24553, 66.1, 45.4, and 56.04, 52.1 mm SL, and (**E, F**) Araranguá River, UFRGS 22964, 61.1 and 60.7 mm SL, respectively, showing a unique color pattern that differs from other congeners in the study area: one layer of coloration composed of not coalescent round small blotches.

Given the morphological and genetic similarity between the populations of *Cambeva balios* from the Laguna dos Patos System (type locality) and the Mampituba River basin with *C. botuvera* (see below) from the Itajaí River basin, we promote a morphometric comparison. A PCA showed that these populations overlap to some degree (Fig. **S2**). More precisely, the populations of *C. balios* from Laguna dos Patos and *C. botuvera* from the Itajaí River basin overlap at a higher level than the population of *C. balios* from the Mampituba River basin. This fact is mainly explained by the high values of PC1 presented in the *C. balios* population from Mampituba River basin associated with longer barbels length (*i.e*., nasal, maxillary, and rictal). In sum, PC1 explains 44.69% and PC2 20.23% of the variation observed in the analyzed specimens.

Cambeva guaraquessaba and *C. tupinamba* are genetically similar (see below), but the PCA (Fig. **S2**) showed a separation between them associated mainly with the supra-orbital pore distance. PC1 explains 32.70% and PC2 22.45% of the variation observed in the analyzed specimens.

Phylogenetic reconstruction. We investigated the phylogenetic relationships of *Cambeva* species using a mitochondrial matrix of COI (597 bp) under a Bayesian inference. The best models for nucleotide substitution estimated by codon position in PartitionFinder for the BEAST analyses were TrNef + I, HKY and GTR+G, respectively. The phylogenetic analysis of *Cambeva* (Fig. 11; Fig. **S7**) resulted in three large species-inclusive clades with relative high support (posterior probability values - PP): named here in as clade "A" (PP = 0.99), clade "B" (PP = 0.94), and clade "C" (PP = 1). The clade A includes a subclade (PP = 0.99) containing *C. brachykechenos* (Ferrer & Malabarba, 2013), *C. poikilos* (Ferrer & Malabarba, 2013), *C. perkos* (Datovo, Carvalho & Ferrer, 2012), and *Cambeva* sp. 1 sister-group to another subclade (PP = 0.41) composed by *C. balios*, *C. botuvera*, *C. diatropoporos* (Ferrer & Malabarba, 2013), and *C. tropeiro* (Ferrer & Malabarba, 2011). In clade A, samples belonging to the morphologically delimited species *C. balios*, *C. botuvera*, *C. perkos*, and *Cambeva* sp. 1 were recovered as non-monophyletic. *Cambeva balios* plus *C. botuvera* form a well-supported monophyletic group sister-group to *C. tropeiro* (PP = 1).

The Clade B includes *C. barbosae*, *C. cubataonis*, *C. davisi*, *C. diabola* (Bockmann, Casatti & de Pinna, 2004), *C. flavopicta* Costa, Feltrin & Katz, 2020, *C. horacioi* Reis, Frota, Fabrin & Graça, 2019, *C. naipi* (Wosiacki & Garavello, 2004), *C. pascuali* (Ochoa, Silva, Costa e Silva, Oliveira & Datovo, 2017), *C. perkos*, *C. stawiarski* (Miranda Ribeiro, 1968), *C. zonata*, and *Cambeva* sp. 2. Internal relationships in Clade B are generally poorly resolved (low supports) and several species are not monophyletic, including those from coastal drainages, excepting *Cambeva* sp. 2, which has two populations (Araranguá and Tubarão river basins) reciprocally monophyletic (PP = 1). Populations of *C. barbosae* are nested within *C. cubataonis sensu* Katz *et al*. (2018) and *C. diabola sensu* Pereira *et al*. (2013) (PP = 0.6), being sister group to *C. davisi sensu* Nascimento *et al*. (2017) plus *C. perkos sensu* Donin *et al*., 2020 (PP = 1). Specimens representing *C. cubataonis* form a monophyletic group (PP = 1) excluding the likely misidentified specimens of *C. cubataonis sensu* Katz *et al*. (2018). *Cambeva zonata* is nested within a lineage along with *Cambeva davisi sensu* Morais-Silva *et al*. (2018). *Cambeva davisi* were recovered as polyphyletic, appearing independently in several portions of the clade B. Specifically, the population of *C. davisi* (*sensu* Nascimento *et al*., 2017) from the Ribeira de Iguape River basin is inserted in a lineage with *Cambeva stawiarski sensu* Donin *et al.* (2020) and

C. davisi sensu Ochoa *et al*. (2017a) and Morais-Silva *et al*. (2018) from upper Paraná and Iguaçu river basins (PP = 1).

The Clade C contains *C. iheringi*, *C. guaraquessaba*, *C. tupinamba*, *C. variegata* (Costa, 1992), which were recovered as non-monophyletic except for the latter with a single sample in the analysis. *Cambeva guaraquessaba* and *C. tupinamba* form a highly supported monophyletic group (PP = 1) sister-group of a clade of *C. iheringi* represented by samples from continental and coastal drainages (PP = 0.96).

Species delimitation and congruence. The mitochondrial COI (597 bp), was used for the species delimitation analyses (GMYC, bPTP; **S1**). Best-fit models of nucleotide substitution estimated for the non-ultrametric for bPTP tree were TIM+F+I+G4. We conducted the GMYC analysis using distinct ultrametric reconstruction in BEAST resulted from a combination of distinct priors (combinations 1, 2, 3, and 4; *i.e*., clock models and tree priors).

The GMYC results differed significantly depending on the prior combination used for the phylogenetic reconstruction (Fig. 11; Tab. 2; Figs. **S3–S7**), and none was substantially congruent with the morphological assessment. In these analyses, 2, 5, 5, and 43 groups were suggested (Tab. 2; Figs. **S3–S7**) with large confidence intervals in the number of clusters.

Combination 1 (relaxed clock lognormal/yule model) has only two clusters (ranging from 1 to 23; Tab. 2; Fig. **S3**), groups all species of clade A in one cluster and all species from clades B and C on another. Combination 2 (relaxed clock -lognormal /coalescent constant population size) supports five cluster (from 2 to 45; Tab. 2; Fig. **S4**). This combination groups clade A into 2 clusters: the first formed by *Cambeva tropeiro* and *C. balios*, and the second by *C. brackykechenos*, *C. diatropoporos*, *C. perkos* (Datovo, Carvalho & Ferrer, 2012), *C. poikilos*, and the undescribed species *Cambeva* sp. 1; clade B into two clusters, one containing *Cambeva* sp. (= *C.* sp.; two juvenile specimens with uncertain morphological identification), *C. davisi*, *C. flavopicta*, *C. horacioi*, *C. pascuali*, *C. taroba* (Wosiacki & Garavello, 2004), *C. zonata*, and *Cambeva* sp. 2; another with *C. barbosae*, *C. cubataonis*, *C. davisi*, *C. diabola* (Bockmann, Casatti & de Pinna, 2004), *C. perkos* and *C. stawiarski*; and the last cluster formed by species from clade C (*C. guaraquessaba*, *C. iheringi*, and *C. tupinamba*). *Cambeva naipi* is suggested as a single entity.

Combination 3 (strict clock/yule model) presents the same groupings of the combination 2 (ranging from 1 to 43; Tab. 2; Fig. **S5**), except for *Cambeva* sp. 2, which groups with *C. barbosae*, *C. cubataonis*, *C. davisi*, *C. diabola*, *C. naipi*, *C. perkos*, and *C. stawiarski*.

Combination 4 (strict clock/coalescent constant population size) is the one that generated the largest number of clusters (43 with confidence interval from 5 to 46; Tab. 2; Figs. **S6** and **S7**). Likelihoods for the cutoff point in the GMYC analyses were similar throughout most of the diversification of *Cambeva* in all combinations examined (Tab. 2). Fitting of the empirical data on the GMYC model using the parametric bootstrap test of the P2C2M. GMYC analysis indicates that the model was violated (p-value = 0; threshold = 0.1) in three of the four phylogenetic reconstructions using the distinct priors. The GMYC model was not violated (p-value = 0.99; threshold = 6.7) only in the analysis where the input tree was reconstructed using a combination of a strict molecular clock and a coalescent tree prior of coalescent constant population size (Fig. 11; Figs. **S6** and **S7**).

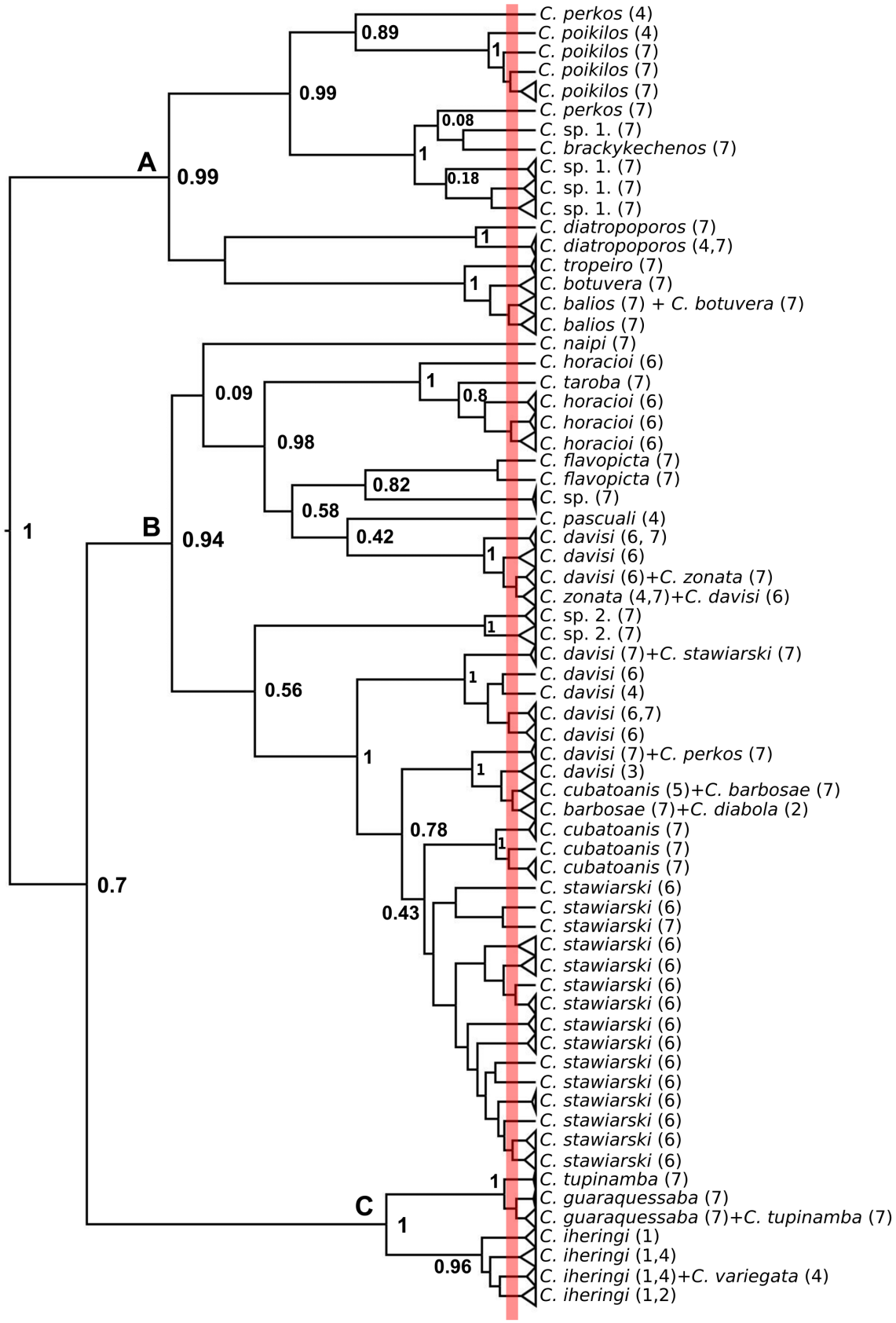

FIGURE 11 | Bayesian phylogenetic tree of *Cambeva* species obtained from COI data. The vertical red line shows the coalescent branching process estimated by using the single-threshold model in the GMYC combination 4 (strict clock model *vs.* a coalescent constant population size tree prior).

TABLE 2 | Comparative results of GMYC species delimitation for each Clock/Prior combination tested.

Combinations	1	2	3	4
Clock Model/Prior	Relaxed/Yule	Relaxed/CCP	Strict/Yule	Strict/CCP
Likelihood of null model	804.0655	835.278	800.5291	834.1315
Maximun likelihood of GMYC model	804.5015	838.5136	802.0595	839.2084
Likelihood ratio	0.8719561	6.471159	3.060767	10.15375
Number of ML clusters	2	5	5	43
Confidence interval	1-23	2-45	1-43	5-46
Number of ML entities	2	6	5	67
Confidence interval	1-169	2-94	1-169	5-84
Threshold time	-1.470165	-1.664397	-1.364488	-0.08717104

The PTP results support the presence of 13 clusters and 8 single entities to the maximum likelihood (mPTP) (Fig. **S8**); and average 35 clusters and 77 single entities, respectively, for the Bayesian analysis (bPTP) (Fig. **S9**). The estimated number of species ranges from 61 to 129, with a mean of 98. Most of the PTP results only partially corroborate the morphological delimitation of the coastal species of *Cambeva*. The mPTP corroborates only the morphologically delimited species *Cambeva* sp. 1 and *Cambeva* sp. 2 (Fig. S8). The bPTP results support either clustering of morphologically delimitated species (*e.g.*, *C. zonata* with *C. davisi*) or splitting within the same morphospecies (*e.g.*, *Cambeva* sp.1) (Fig. **S9**). These two analyses are largely incongruent (Figs. **S8** and **S9**).

DISCUSSION

Species delimitation methods. Popular methods of species delimitation based on single-locus data like bPTP and GMYC are widely used in biodiversity assessments in the Neotropical ichthyofauna (García-Melo *et al*., 2019; Agudelo-Zamora *et al*., 2020; Delapieve *et al*., 2020, Mateussi *et al*., 2020). We recognize that these methods can be a crucial exploratory tool in recognizing species. However, it must be done with caution. Our results indicated that, based on the dataset used, the species delimitations within *Cambeva* were highly variable between the used methods and mostly incongruent with the morphological delimitation. Within the GMYC, we also tested the impact of the different priors on grouping morphospecies (Talavera *et al*., 2013; Luo *et al*., 2018), resulting in different boundaries on each prior tested, ranging widely from 2 to 43 clusters. These results go against what was found by Talavera *et al*. (2013) that the results did not indicate substantial differences in the number of clusters found using input trees recovered by Coalescent or Yule priors and relaxed or strict molecular clocks.

The evaluation of the statistical fit of GMYC method shows that the model was violated in most of the phylogenetic reconstructions using the distinct priors (except for the combination of strict clock model with a coalescent constant population size tree prior). Simulation testing demonstrates that the underlying process of diversification and effective population size influences how well the GMYC model fits a given dataset (Luo *et al*., 2018; Fonseca *et al*., 2021). At small values of effective population size, allele coalescence occurs faster than speciation, being straightforward to model the differences between these processes. Taking geographic distribution on distinct and isolated basins as a proxy for effective population size, *Cambeva* shows a combination of widespread species presenting genetically structured populations in isolated basins (*e.g*., *C. barbosae*, *C. cubataonis*, *Cambeva* sp. 1, *Cambeva* sp. 2) but also species restricted to small areas in single basins (*e.g*., *C. tropeiro* and *C. zonata*). Other demographic scenarios that affect GMYC model fit are population expansion and geographic structure within species. In *Cambeva*' species, faunal interchange between coastal and continental basins may trigger rapid population expansion (*e.g*., *Cambeva* sp. 1).

In addition to the violation of the GMYC model, the disagreement between the methods tested suggests that the analysis used here based on single-locus data is likely not adequate to assess the species limits within *Cambeva*. This failure can also be attributable to the intrinsic COI polymorphism properties, such as overlap in inter- and intraspecific divergences and non-monophyly of the species likely because of introgression or incomplete lineage sorting) (Knowles, Carstens, 2007; Talavera *et al*., 2013). Additionally, the performance of these single-locus-based methods strongly decreases when population sizes and speciation rates are high (Dellicour, Flot, 2015), and, in these cases, multilocus approaches are recommended. For the GMYC model, for example, a certain lumping tendency has been demonstrated and attributed to the poorly model ability to work process as incomplete lineage sorting or clades undergoing rapid radiation (Esselstyn *et al*., 2012; Reid, Carstens, 2012; Talavera *et al*., 2013). In our dataset, such a tendency was observed in the clade C with lumping between *C. guaraquessaba* and *C. tupinamba* in both GMYC and bPTP analyses.

Currently, studied introgression (hybridization) cases between species or genetically structured populations are rare in Neotropical fishes (*e.g*., Willis *et al*., 2012 in *Cichla* Bloch & Schneider, 1801; Sales *et al*., 2018 in *Prochilodus* Agassiz, 1829; Barreto *et al*., 2020 in *Nematocharax* Weitzman, Menezes & Britski, 1986). Events of hybridization were proposed between genetically structured populations of *Nematocharax* among isolated river basins in coastal rivers of Eastern Brazil (Barreto *et al*., 2020). These events were associated with capture stream events of populations in the headwaters of these basins. This type of hybridization event associated with rearrangement of drainage basins may be the case of *Cambeva* populations that inhabit headwaters and often occur in sympatry.

Inferences regarding species boundaries based on genetic data alone need to be analyzed carefully, and we reinforce those inferences of species boundaries should be conducted with consideration of other data sources, such as morphology, in an integrative approach (Pereira *et al*., 2013; Camelier *et al*., 2018; Serrano *et al*., 2019). Additionally, we suggest considering factors that can affect the efficiency of the delimitation method used for a given dataset, and we reinforce the need to test the dataset and models used in these analyzes (*i.e*., Fonseca *et al*., 2021), which is rarely done in these species delimitation analyses.

In this sense, we observe in the literature that incongruence across the results from different species delimitation methods are relatively common, and inferences and taxonomical acts drawn from these results are often not conservative. Thereby, in most contexts, it is better to fail to delimit species than it is to falsely delimit entities that do not represent independent evolutionary lineages (Carstens *et al*., 2013).

***Cambeva* taxonomy and distribution.** Trichomycterinae is the most species-rich group of Trichomycteridae with 270 species, and by far, the one with more recent additions, with 93 species described in the last decade (Fricke *et al*., 2022). Several new species have been proposed in Trichomycterinae in the last years, but some species remain poorly defined. One of the caveats is that species are described based on a few specimens (*e.g*., < 10) and from limited localities, many of them, known from the type locality only (Wosiacki, 2005; Costa *et al*., 2020a,b; Katz, Costa, 2020), limiting assessment of intraspecific morphological and molecular variation. Contemplating a wide geographic sampling range and specimens of varied sizes are the main recommendations to properly investigate intraspecific color variation (Menezes *et al*., 2015; Nascimento *et al*., 2017), which deserves to be highlighted. Specifically, in Trichomycterinae, this feature has been extensively demonstrated to be associated with ontogeny (Castellanos-Morales, 2007; Lima *et al*., 2008; Ferrer, Malabarba, 2013; Nascimento *et al*., 2017); microhabitat preference (Arratia *et al*., 1978; Arratia, Menu-Marque, 1981; Arratia, 1983) or both (Silva *et al*., 2010).

The species richness of *Cambeva* has also increased substantially, with more than half (26) of the 46 valid species described in the past ten years. *Cambeva* is a dominant genus among the restricted-range species from Brazil (Nogueira *et al*., 2010) with many species restricted to the upper portions of drainages. In fact, our review demonstrated that there are restricted-range examples along the study area (*e.g*., *C. guaraquessaba* and *C. zonata*), but the most species have wider distribution than previously supposed (Fig. 1). However, the most notable distribution extensions were demonstrated in species recently described or redescribed: *Cambeva barbosae* to Tijucas and Cubatão Norte basins; *C. botuvera* to a wide area along the Itajaí River basin; and *C. cubataonis* to Babitonga, Guaratuba, and Paranaguá bays. Curiously, these species have the highest levels of color variation in the study area (Figs. 4–6; Tab. 1), which also were poorly explored in their descriptions. In addition, *C. barbosae* and *C. cubataonis* are sympatric in the Itapocu and Cubatão Norte river basins, a condition not noticed by Costa *et al*. (2021) and Katz, Barbosa (2014).

The coloration of *C. barbosae* and *C. cubataonis* are usually similar, with the former composed of irregular and coalescent inconspicuous black marks or sparse blotches (Fig. 4) and the latter ranging from a mottled pattern of coalescent black blotches to a body almost entirely black (Fig. 6). For now, we assume that these color variations are not associated with ontogeny since we evaluate individuals of varied sizes, but preferences and association of individuals or populations with specific microhabitats deserve further investigation (Donin *et al*., 2020).

The intraspecific variation in the coloration is even more pronounced in *C. botuvera*, which has two distinct color layers in adult specimens composed of large ellipsoid black blotches, sometimes coalescent and forming a mid-lateral stripe, vermicular black marks, or yet inconspicuous black-spotted in the inner layer, and small spots in the outer layer (Fig. 5). *Cambeva balios* also has two distinct layers of coloration with

similar variations (see Ferrer, Malabarba, 2013) and the only diagnostic characters from *C. botuvera* are the number of procurrent rays (Tab. 1). Considering these often-intraspecific variable morphological differences between the two species in addition to their almost indistinguishable coloration with layers of rounded blotches and genetic similarity, the validity of *C. botuvera* needs a more extensive evaluation.

The current morphological delimitation provided was conducted based on the diversity of *Cambeva* found in the study area and the species diagnosis are restricted to a sample of characters (Tab. 1). Given that, the present work does not aim to propose new diagnosis or recommend their broad usage within the genus. In the meantime, we recognize that some diagnosis, descriptions, and geographic delimitation for the taxa analyzed are current insufficient and a taxonomic review in the area in progress. In addition, considering all incongruences in the species delimitation methods and the high intraspecific variability found in *Cambeva*' species, we reinforce the search and combination of multiples evidences to support hypotheses of species in this complex group.

Material examined. Lots and information in addition to those cited in **Donin *et al.* (2020) and Ferrer, Malabarba (2020).** "c&s" **= specimens** cleared and counterstained; "tis" = samples tissues followed by their collection codes. All from Brazil. ***Cambeva balios*: Taquari-Antas River basin.** UFRGS 16233, 3 (1 c&s), paratypes, 47.1–81.9 mm SL; UFRGS 16341, 3 (1 c&s), paratypes, 35.2–67.2 mm SL. **Uruguai River basin:** UFRGS 21886, 9 (tis TEC 6861 A, B), 19.6–74.4 mm SL. **Mampituba River basin.** UFRGS 16295, 10 (2 c&s), 48.6–80.1 mm SL; UFRGS 16299, 2, 41.3–60.8 mm SL; UFRGS 16284 (tis TEC 2738 A, B), 2, 27.5–28.5 mm SL; UFRGS 17198 (tis TEC 3203), 1, 59.4 mm SL. ***Cambeva barbosae*: Cubatão Sul River basin.** MCP 16570, 1, 32.4 mm SL; MCP 17477, 2, 31.9–57.5 mm SL; UFRGS 22906, 17 (tis TEC 7344 A, B; 2 c&s), 33.5–70.6 mm SL; UFRGS 24296, 6, 28.1–56.2 mm SL. **Biguaçu River basin.** UFRGS 22907, 2 (tis TEC 7345 A, B), 36.5–61.3 mm SL; UFRGS 22932, 6 (tis TEC 7360 A, B; 1 c&s), 36.5–66.5 mm SL. **Tijucas River basin.** UFRGS 20914, 1, 50.9 mm SL; UFRGS 20915, 2 (1c&s), 41.1–49.8 mm SL; UFRGS 23235, 1 (tis TEC 7458), 24.3 mm SL. **Santa Catarina Island.** UFRGS 23183, 4 (tis TEC 7447 A, B), 49.1–80.0 mm SL. **Itapocu River basin.** MCP 32214, 3, 57.5–62.9 mm SL; MCP 32190, 1, 44.9 mm SL; MNRJ 38421, 1, 63.1 mm SL; MNRJ 52565, 2, 45.3–47.3 mm SL; NUP 14240, 1, 53.5 mm SL; UFRGS 18481, 1, 54.1 mm SL; UFRGS 28701, 3, 43.5–51.2 mm SL. **Cubatão Norte River basin:** MCP 50476, 1, 52.0 mm SL; MCP 50485, 1, 42.6 mm SL; MNRJ 13191, 1, 39.1 mm SL; MNRJ 40952, 7, 43.4–52.6 mm SL; UFRGS 28698, 4, 41.3–71.2 mm SL; UFRGS 28697, 2 (1 c&s), 44.1–53.6- mm SL; UFRGS 28699, 27 (1 c&s), 22.7–54.1 mm SL. ***Cambeva botuvera*: Itajaí River basin.** UFRGS 20118, 8, 25.2–111.9 mm SL; UFRGS 22939 (tis TEC 7365 A, E; 2 c&s), 25, 37.9–78.8 mm SL; UFRGS 23182 (tis TEC 7446 A, C, F, I, L, M, P; 1 c&s), 27, 33.6–104.4 mm SL; UFRGS 24527, 1, 68.4 mm SL; UFRGS 24558, 4, 47.4–65.5 mm SL. ***Cambeva brackykechenos*: Sinos River basin.** UFRGS 24666, 1 (tis TEC 8229), 43.3 mm SL. ***Cambeva cubataonis*: Itapocu River basin.** MNRJ 41029, 1, 66.4 mm SL; UFRGS 24449, 1, 68.4 mm SL. **Cubatão Norte River basin.** UFRGS 24311, 2 (tis TEC 7996 A), 47.18–86.05 mm SL; UFRGS 24543, 6 (tis TEC 8117 A; 1 c&s), 32.7–76.8 mm SL; UFRGS 24567, 2 (1 c&s), 51.5–55.5 mm SL. **Babitonga Bay.** UFRGS 24552, 4 (tis TEC 8126 A, B; 1 c&s), 61.6–85.7 mm SL. **Guaratuba Bay.** UFRGS 24549, 8 (1 c&s), 53.1–64.7 mm SL; UFRGS 24556, 5 (tis TEC 8130 A, B), 45.3–62.9 mm SL. **Paranaguá Bay:**MNRJ 32940, 1 (tis MNLM 4644), 57.0 mm SL. ***Cambeva davisi*: Upper Paraná River basin.** MZUEL 11776, 10 (2 c&s), 30.8–92.2 mm SL. **Iguaçu River basin.** MZUEL 14788 (tis A, B), 2, 42.4-55.9 mm SL. **Ribeira de Iguape River basin.** MZUEL 17202 (tis A, B), 4, 38.3–73.1 mm SL; MZUEL 17213 (tis A, B), 2, 45.4–50.5 mm SL. ***Cambeva diatropoporos*: Taquari-Antas River basin.** UFRGS 17887, 1 (tis TEC 3560), 59.1 mm SL; UFRGS 17889, 1 (tis TEC

3562), 34.2 mm SL. ***Cambeva flavopicta*: Uruguai River basin.** MCP 17440, 5 (1 c&s), 29.9–61.5 mm SL; UFRGS 3964, 1, 29.9 mm SL; UFRGS 14998 (tis TEC 1780 A, B), 2, 28.7–30.2 mm SL; UFRGS 15000, 15, 21.6–42.2 mm SL. ***Cambeva guaraquessaba*: Paranaguá Bay.** MNRJ 40846, 11 (tis 7152), 53.8–36.5 mm SL; UFRGS 24541, 1 (tis TEC 8115 A), 30.88 mm SL; UFRGS 24554, 4 (tis TEC 8128 A), 39.4–46.7 mm SL. ***Cambeva iheringi*:** MNRJ 20129, 3, 36.3–44.2 mm SL. ***Cambeva naipi*: Iguaçu River basin.** UFRGS 11405, 1 (tis TEC 6739 A), 42.2 mm SL. ***Cambeva pascuali*: Paranapanema River basin.** MZUSP 121681, holotype, 49.1 mm SL. ***Cambeva perkos*: Paranapanema River basin.** MZUSP 82372, 1 paratype, 74.4 mm SL.Ijuí River basin UFRGS 17923 (tis TEC 3596 A), 1, 58.9 mm SL; UFRGS 18252 (tis TEC 3749 A), 1, not measured. ***Cambeva poikilos*: Jacuí River basin.** UFRGS 15014, 1 (tis TEC 1795 A), 56.3 mm SL; UFRGS 20352, 1 (tis TEC 5622 A), 60.2 mm SL. **Sinos River basin.** UFRGS 17419, 1 (tis TEC 3314 A), 59.2 mm SL; **Taquari-Antas River basin.** UFRGS 24604, 1 (tis TEC 8177 A), 54.3 mm SL. ***Cambeva stawiarski*: Iguaçu River basin.** UFRGS 18307, 10 (tis TEC 3793 A, B; 2 c&s), 31.7–49.4 mm SL. ***Cambeva taroba*: Iguaçu River basin.** UFRGS 18308, 1 (tis TEC 3794 A), not measured. ***Cambeva tupinamba*: Ribeira de Iguape River basin.** MZUSP 83727, 2, 61.0–64.4 mm SL; UFRGS 25094, 2, 52.8–70.4 mm SL. UFRGS 24550, 2 (tis TEC 8124 A, B), 39.8–62.1 mm SL; UFRGS 24563, 2 (tis TEC 8137 A, B), 22.7–43.4 mm SL. ***Cambeva tropeiro*: Antas River.** UFRGS 21878, 2 (tis TEC 6855 A, B), 81.1–83.2 mm SL. ***Cambeva zonata*: Ribeira de Iguape River basin.** UFRGS 18675, 1 (tis TEC 3998 A), 48.2 mm SL; UFRGS 24538, 11 (tis TEC 8112 A, B), 47.1–61.6 mm SL. ***Cambeva* sp. 1: Tramandaí River basin.** MCN 18587, 1, 62.7 mm SL; MCP 25375, 1, 47.0 mm SL; MCP 25395, 1, 52.0 mm SL; MCP 29117, 1, 46.1 mm SL; MCP 29143, 1, 32.2 mm SL; MCP 29147, 1, 38.6 mm SL; MCP 29694, 1, 20.7 mm SL; UFRGS 10651, 1 (c&s), 66.5 mm SL; UFRGS 17575, 1, 58.60 mm SL; UFRGS 19149, 2 (tis TEC 4867 A, B), not measured; UFRGS 19169, 1 (c&s), 61.4 mm SL; UFRGS 19178, 1 (tis TEC 4881 A), 55.3 mm SL; UFRGS 21346, 1, 52.3 mm SL; UFRGS 22211, 1, 58.6 mm SL; UFRGS 23494, 1 (tis TEC 7583 A), 53.0 mm SL. **Mampituba River basin.** MCP 23623, 2; 51.7–55.7 mm SL; UFRGS 17220, 2 (tis TEC 3225 A, B), 51.0–55.2 mm SL; UFRGS 17228, 1 (tis TEC 3233 A), 46.6 mm SL; UNICTIO 906, 1, 60.2 mm SL. **Araranguá River basin.** MCP 10646, 5, 51.6–20.4 mm SL; MZUEL 07547, 1, 39.2 mm SL; UFRGS 22962, 6 (2 tis TEC 7378 A, B; 1 c&s), 27.7–59.8 mm SL; UNICTIO 2460, 2, 53.2-60.8 mm SL. ***Cambeva* sp. 2: Tubarão River basin.** CPUFMT 79, 11, 12.7–18.5 mm SL; CPUFMT 86, 2, 22.6–28.3 mm SL; CPUFMT 106, 1, 36.1 mm SL; UFRGS 23184, 2 (tis TEC 7448 A, B), 31.2–51.8 mm SL; UFRGS 23185, 3 (tis TEC 7449 A; 1c&s), 33.8–59.8 mm SL; UFRGS 24553, 12 (1 c&s), 25.9-65.7 mm SL. **Araranguá River basin.** MCP 10642, 10, 28.0–49.6 mm SL; UFRGS 22964, 7 (tis TEC 7380 A, B; 2 c&s), 41.2–61.0 mm SL. **Itajaí River basin**: UFRGS 22939, 2 (tis TEC 7365 F, I), 35.3–45.1 mm SL. ***Ituglanis amphipotamus*: Ribeira de Iguape River basin.** MZUSP 69393, holotype, 72.5 mm SL. ***Ituglanis australis*: Uruguay River basin.** MZUSP 112505, holotype, 56.4 mm SL. ***Ituglanis bambui*: Tocantins River basin.** MZUSP 79860, holotype, 42.1 mm SL. ***Ituglanis boitata*: Tramandaí River basin.** UFRGS 18455, holotype, 102.9 mm SL. **Tubarão River basin.** UFRGS 22903, 3, 45.8–63.4 mm SL; UFRGS 24547, 3, 58.3–91.7 mm SL. ***Ituglanis goya*: Tocantins River basin.** MZUSP 119759, holotype, 66.8 mm SL. ***Ituglanis inusitatus*: Uruguai River basin.** UFRGS 21829, holotype, 62.2 mm SL. ***Trichomycterus astromycterus*: Doce River basin.** MZUSP 124559, holotype, 51.1 mm SL. MZUSP 123760, 5 paratypes, 33.2-52.3 mm SL. ***Trichomycterus dali*: Paraguai River basin.** MZUSP 106630, holotype, 76.9 mm SL. ***Trichomycterus itacarambiensis*: São Francisco River basin.** MZUSP 50548, 4 paratypes, 21.1–47.3 mm SL. ***Trichomycterus jacupiranga*: Ribeira de Iguape River basin.** UFRGS 24555, 2 (tis TEC 8129 A, B), 35.1–44.8 mm SL. ***Trichomycterus payaya*: Itapicuru River basin.** MZUSP 88164, 4, paratypes, 33.5–36.3 mm SL.

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AUTHORS' CONTRIBUTION

Laura M. Donin: Conceptualization, Data curation, Formal analysis, Writing-original draft, Writing-review and editing.

Juliano Ferrer: Conceptualization, Data curation, Formal analysis, Writing-review and editing.

Tiago P. Carvalho: Conceptualization, Data curation, Formal analysis, Writing-review and editing.

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The authors declare no competing interests.

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