Neotropical Ichthyology

Original article

https://doi.org/10.1590/1982-0224-2024-0040

DNA barcode shows discordant cases among morphological and molecular species identification in *Isbrueckerichthys* (Siluriformes: Loricariidae)

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Isbrueckerichthys is a genus of armored catfish (Siluriformes: Loricariidae) with five species in the lowlands from the Ribeira de Iguape basin and in the uplands of the Tibagi River basin. Despite the validation of the morphological species, molecular data to investigate gene flow and species delimitation have not been completed for all species. For this purpose, we compared sequences of *COI* region associated with barcoding molecular identification, aiming for a species delimitation analysis and generating population data of *I. alipionis*, *I. epakmos*, *I. duseni*, *I.* cf. *duseni*, *I. saxicola*, and *I. calvus*. The K2P genetic distance, molecular species delimitation analysis, and well-sustained branches in the phylogenetic tree validate *I. alipionis*, *I. epakmos*, and *I. duseni*, and suggest *I.* cf. *duseni* as a valid molecular operational taxonomic unit. However, no differences were detected between *I. saxicola* and *I. calvus*. The discordance between molecular and morphological species may be due to the recent dispersal of some *Isbrueckerichthys* representatives at the border between the Ribeira de Iguape and Tibagi basins. The isolation features of the mountainous region of Ribeira de Iguape basin and headwaters captures to uplands are presented to explain the dispersion and the cases of incipient speciation in *Isbrueckerichthys* lineages.

Keywords: Incipient speciation, Integrative taxonomy, Molecular operational taxonomic units, Molecular species delimitation, Systematics.

Submitted May 7, 2024
Accepted August 5, 2024
by Hernán López-Fernández
Epub October 18, 2024

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Online version ISSN 1982-0224
Print version ISSN 1679-6225

Neotrop. Ichthyol.
vol. 22, no. 3, Maringá 2024

Isbrueckerichthys é um gênero de cascudinhos (Siluriformes: Loricariidae) que reúne cinco espécies morfológicas nas terras baixas da bacia do Ribeira de Iguape e nas terras altas da bacia do rio Tibagi. Apesar da validação das espécies morfológicas, estudos moleculares não tinham sido realizados para investigar o fluxo gênico e delimitação molecular das espécies. Para tanto, comparamos sequências da região *COI* associadas à identificação molecular por código de barras visando uma análise de delimitação de espécies e dados populacionais entre *I. alipionis*, *I. epakmos*, *I. duseni*, *I.* cf. *duseni*, *I. saxicola* e *I. calvus*. Os dados de distância genética K2P, a análise de delimitação molecular de espécies e ramos bem sustentados na árvore filogenética validam *I. alipionis*, *I. epakmos* e *I. duseni*, e sugere *I.* cf. *duseni* como unidade taxonômica operacional molecular. Ainda, não foram observadas diferenças entre *I. saxicola* e *I. calvus*. A discordância entre espécies moleculares e morfológicas pode ser devida à recente dispersão e diversificação de alguns representantes de *Isbrueckerichthys* na fronteira das bacias de Ribeira de Iguape e Tibagi. Características de isolamento da região serrana da bacia do Ribeira de Iguape e eventos de capturas de cabeceiras para terras altas foram utilizadas para explicar a dispersão e os casos de especiação incipiente em algumas linhagens de *Isbrueckerichthys*.

Palavras-chave: Delimitação molecular de espécies, Especiação incipiente, Sistemática, Taxonomia integrativa, Unidade taxonômica operacional molecular.

INTRODUCTION

Loricariidae is one of the most species-rich fish families, with 1,056 valid species grouped in 115 genera (Fricke *et al*., 2024). The broad species diversification in Loricariidae makes it difficult for accurate determination of species relationships, leading to doubts in subfamilies, tribes, and genera (Isbrücker, 1980; Armbruster, 2004; Reis *et al*., 2006; Lujan *et al*., 2015; Covain *et al*., 2016; Pereira, Reis, 2017; Roxo *et al*., 2019). Another crucial problem in Loricariidae is the taxonomic uncertainties that occur in paired species belonging to isolated hydrographic basins (Anjos *et al*., 2021; de Sousa *et al*., 2021; Lustosa-Costa *et al*., 2022) or species resident in tributaries and with low vagility (Albert *et al*., 2020; de Sousa *et al*., 2021).

The genus *Isbrueckerichthys* (Loricariidae: Siluriformes) was proposed by Derijst (1996), justified on the discovery of an earlier type-species designation by Regan (1920), where *Pareiorhaphis duseni* (Miranda Ribeiro, 1907) and *Pareiorhaphis alipionis* (Gosline, 1947) were moved to the new genus. Their representatives are small-sized species of armored catfishes distinct from the sister taxon *Neoplecostomus* Eigenmann & Eigenmann, 1888 by several diagnostic morphological characteristics, such as a small naked area behind the pterotic-supracleithrum, abdomen with small platelets embedded in the skin between pectoral girdle and pelvic-fin insertions, one spine and seven branched rays in the dorsal fin, caudal peduncle ovoid in cross-section, and lacking a series of papillae on the lower lip of the dentaries (Derijst, 1996; Pereira, Oyakawa, 2003; Jerep *et al*., 2006). *Isbrueckerichthys* species inhabit headwater streams, usually rapids (<1 m deep), with

well-oxygenated water and substrates consisting of rocks (Pereira, Reis, 2002). The first species recognized as *Isbrueckerichthys* (*I. duseni*, *I. alipionis*, and *I. epakmos* Pereira & Oyakawa, 2003) occur in the plain coastal lands of the Atlantic and are restricted to the Ribeira de Iguape River basin (Derijst, 1996; Pereira, Oyakawa, 2003; Dagosta *et al*., 2024). Afterward, Jerep *et al*. (2006) described two species (*I. saxicola* Jerep, Shibatta, Pereira & Oyakawa, 2006 and *I. calvus* Jerep, Shibatta, Pereira & Oyakawa, 2006) from upland rivers of the Tibagi basin, belonging to the upper Paraná River.

For some authors, a taxonomic system based strictly on morphology underestimates the cryptic species diversity, which is common in many groups (Knowlton, 1993; Jarman, Elliott, 2000; Hebert *et al*., 2003). The essence of this limitation is the phenotypic plasticity and the genetic variability in the characters that can lead to erroneous identifications (Hebert *et al*., 2003). In Neotropical fishes, molecular analyses involving species delimitation or population biology have contributed to the understanding of distribution, taxonomy, and systematics in several groups (do Nascimento *et al*., 2018; Santos *et al*., 2019; Argolo *et al*., 2020; Herrera-Collazos *et al*., 2020; Traldi *et al*., 2020; Ariza *et al*., 2022; Azambuja *et al*., 2022; Silva-Santos *et al*., 2023; Souza *et al*., 2023). Previous studies have stipulated parameters for standardizing values for genetic divergence for molecular markers utilized for species identification (Ward, 2009). For several authors, the interspecific divergence must be ten times higher than the intraspecific divergence for a taxa validation (Hebert *et al*., 2004). Ward (2009) proposed that fish specimens that are strictly related and present genetic divergences above 2% belong to different species. Although the genetic divergence values associated with molecular markers used for molecular identification are standardized for some organisms, it is known that the trigger values for intra/interspecific divergence are variable and are influenced by the variation in the substitution rate through the lineages (Barraclough *et al*., 2009).

In this context, integrative taxonomy/phylogenetics is a contemporary strategy addressed to questions in which single data-based approaches reveal less consistent conclusions rather than those by combined multisource evidence in terms of detecting the species richness within supposedly homogeneous taxa (Padial *et al*., 2010; Schlick-Steiner *et al*., 2010; Grković *et al*., 2017). Distinct species delimitation methods, such as ABGD, ASAP, and bPTP, have been proposed to analyze a single locus (Pons *et al*., 2006; Zhang *et al*., 2013). In general, these analyses have demonstrated that the combination of different methods, as well as integrative taxonomy, allows more representative results for the identification of lineages in species (Tang *et al*., 2014; Kapli *et al*., 2017; Luo *et al*., 2018; Serrano *et al*., 2018; Cardoso *et al*., 2023; Souza *et al*., 2023). *Isbrueckerichthys* is a Loricariidae genus with reformulations and species descriptions in the last three decades (Derijst, 1996; Pereira, Oyakawa, 2003; Jerep *et al*., 2006). However, molecular species delimitation studies are lacking in the genus. Therefore, this study aimed to conduct a molecular delimitation analysis in all *Isbrueckerichthys* species inhabiting the lowlands and uplands in south and southeast Brazilian drainages.

MATERIAL AND METHODS

Biological samples. Specimens of *Isbrueckerichthys* were collected in different tributaries of the lowlands from the Ribeira de Iguape basin and in tributaries of the uplands from the Tibagi basin (Fig. 1). Specimens were deposited as vouchers in the ichthyological collection at Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura of Universidade Estadual de Maringá, Maringá (NUP) and in the Laboratório de Biologia e Genética de Peixes of the Universidade Estadual Paulista "Júlio de Mesquita Filho", Botucatu (LBP) (Tab. 1).

Molecular analyses. Genomic DNA of thirty-one individuals of *Isbrueckerichthys* were extracted from liver samples using the CTAB (cetyltrimethylammonium bromide) method (Murray, Thompson, 1980). The barcode region of the mitochondrial gene cytochrome c oxidase subunit I (*COI*) was amplified by PCR using the primers Fish F1 and Fish R1 (Ward *et al*., 2005). The reaction was composed of 1x *Taq* Reaction buffer (200 mM Tris pH 8.4, 500 mM KCl), 0.2 mM dNTPs, 1 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Invitrogen), 0.4 µM of each primer, and 40 ng of DNA. The following reaction program was used: initial denaturation for 10 min at 94 °C, 35 cycles of 94 °C for 1 min, 51.8 °C for 45 sec and 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were purified with the Illustra GFX PCR DNA and Gel Band Purification (GE Healthcare) and sequenced in an ABI-Prism 3500 Genetic Analyzer (Applied Biosystems).

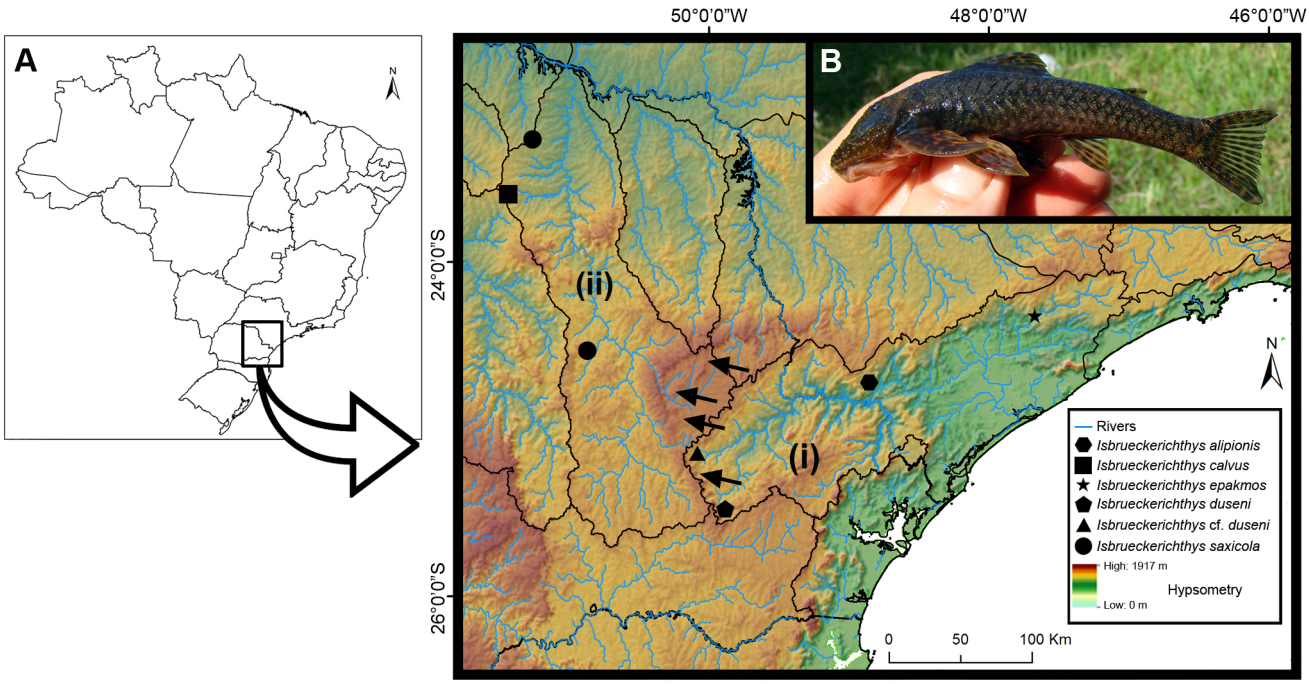

FIGURE 1 | Collection sites and representative image of an *Isbrueckerichthys* species. **A.** Partial map of Brazil highlighting south/southern hydrographic basins of the Ribeira de Iguape River (i) and Tibagi River (ii). The arrows show the Ponta Grossa Arch region in eastern Paraná State, Brazil (a relief barrier between lowlands and uplands hydrographic basins). The map shows elevation and valleys in the relief, and the geometric shapes indicate the collection site of the specimens of *Isbrueckerichthys* analyzed. **B.** Live specimen of *I. calvus* photograph taken during capture in the Juruba stream.

TABLE 1 | Collection sites of the *Isbrueckerichthys* species, number of individuals analyzed, and voucher numbers.

Species	Number of individuals	Samples	Coordinates	Voucher number
Isbrueckerichthys alipionis	7	Betari River/Ribeira de Iguape basin	24°33'42.1"S 48°40'05.7"W	LBP 7373
Isbrueckerichthys calvus	5	Juruba stream (type- locality)/Tibagi basin	23°34'24.68"S 51°23'50.30"W	LBP 6404
Isbrueckerichthys duseni	5	Açungui River/Ribeira de Iguape basin	25°22'43.9"S 49°38'58.9"W	NUP 16200
Isbrueckerichthys cf. *duseni*	8	D'Areia stream/Tibagi basin	25°14'09.0"S 50°00'17.0"W	NUP 20293
Isbrueckerichthys epakmos	3	Juquiá River/ Ribeira de Iguape basin	24°01'25.2"S 47°27'20.3"W	LBP 7385
Isbrueckerichthys saxicola	5	Jacutinga stream (type- locality)/Tibagi basin	23°14'29.87"S 51°13'5.10"W	LBP 40259-40263
	3	Charqueada River/Tibagi basin	24°29'09.0"S 50°43'55.0"W	NUP 20291

Sequences were analyzed using Geneious v. 7.1.9 (Kearse *et al*., 2012) software and submitted to GenBank. Five sequences of *I. saxicola* (GU701901 – GU701903; GU701905 – GU701906) from the Jacutinga River were recovered from the GenBank. The sequences were aligned with the ClustalW algorithm integrated into the Geneious program. Nucleotide (π) and haplotype (Hd) diversity were computed by DnaSP v. 5 (Librado, Rozas, 2009). Sequences were separated into groups, and genetic distances were calculated in MEGA X (Kumar *et al*., 2018) under the Kimura-2-Parameters evolution model and 1,000 bootstrap replications. A haplotype network was generated through the median-joining criterion (Bandelt *et al*., 1999) in the PopArt v. 1.7 software (Leigh, Bryant, 2015). Structural analysis was performed by assigning each individual to the respective populations using Bayesian Analyses of Population Structure – BAPS 6, allowing for a range of 1–6 (Corander *et al*., 2004, 2008). Population structuring and analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) were performed by Arlequin v. 3.5.2.2 software (Excoffier, Lischer, 2010).

One *Pareiorhaphis splendens* (Bizerril, 1995) (Neoplecostomini) COI sequence EU359449.1 was aligned to *Isbrueckerichthys* with ClustalW and used to root trees, according to previous molecular phylogenetic studies (Roxo *et al*., 2012). The alignment was submitted to jModelTest 2 (Darriba *et al*., 2012) using the corrected Akaike information criterion (AICc) to select the best-fit nucleotide evolution HKY model for downstream analyses. A Bayesian inference tree was generated in the MrBayes 3.2 program (Huelsenbeck, Ronquist, 2001; Ronquist, Huelsenbeck, 2003), applying 100,000,000 interactions of MCMC, sampling trees every 10,000 generations and burn-in of 10,000,000.

Three methods for species delimitation were applied: Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al*., 2012), Assemble Species by Automatic Partitioning (ASAP) (Puillandre *et al*., 2020), and Bayesian Poisson Tree Processes (bPTP) (Zhang *et al*., 2013). The alignment generated for the sequences was used as an input file for the delimitation of species in the ABGD web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) and in the ASASP web server (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html), the Kimura (K80) TS/TV was selected for distance mode and the other parameters were kept at default. For bPTP analysis, the Bayesian tree generated

in MrBayes was used as an input file in a bPTP web server (https://species.h-its.org/ptp/). There were 500,000 MCMC generations run (thinning = 500), and the other parameters were kept at default.

RESULTS

Sequences of the COI gene were obtained from the *Isbrueckerichthys* specimens and deposited in GenBank (PP391830–PP391860). All sequences were of high quality and presented no evidence of insertions, deletions, or stop codons. The obtained sequences were aligned with the sequences of *I. saxicola* from the GenBank database, and a matrix of 36 sequences with 612-bp was generated. The nucleotide diversity (π) was 0.01369, and haplotype diversity (Hd) was 0.806. A total of ten haplotypes were observed: *Isbrueckerichthys alipionis* had four haplotypes; *I. calvus* and *I. saxicola* shared a unique haplotype; individuals of *I. duseni* and *I.* cf. *duseni* had one exclusive haplotype to each species; and in *I. epakmos* three haplotypes were observed (Fig. 2A).

The intraspecific distance for each group ranged from 0 to 0.23%, and the interspecific distance ranged from 0 to 3.63% (Tab. 2). The BAPs results indicated an optimal partition of four clusters (k = 4; marginal log-likelihood = -240.4536). Individuals of *I. calvus* and *I. saxicola* were assigned as a single group, *I. duseni*, and *I.* cf. *duseni* were separated into

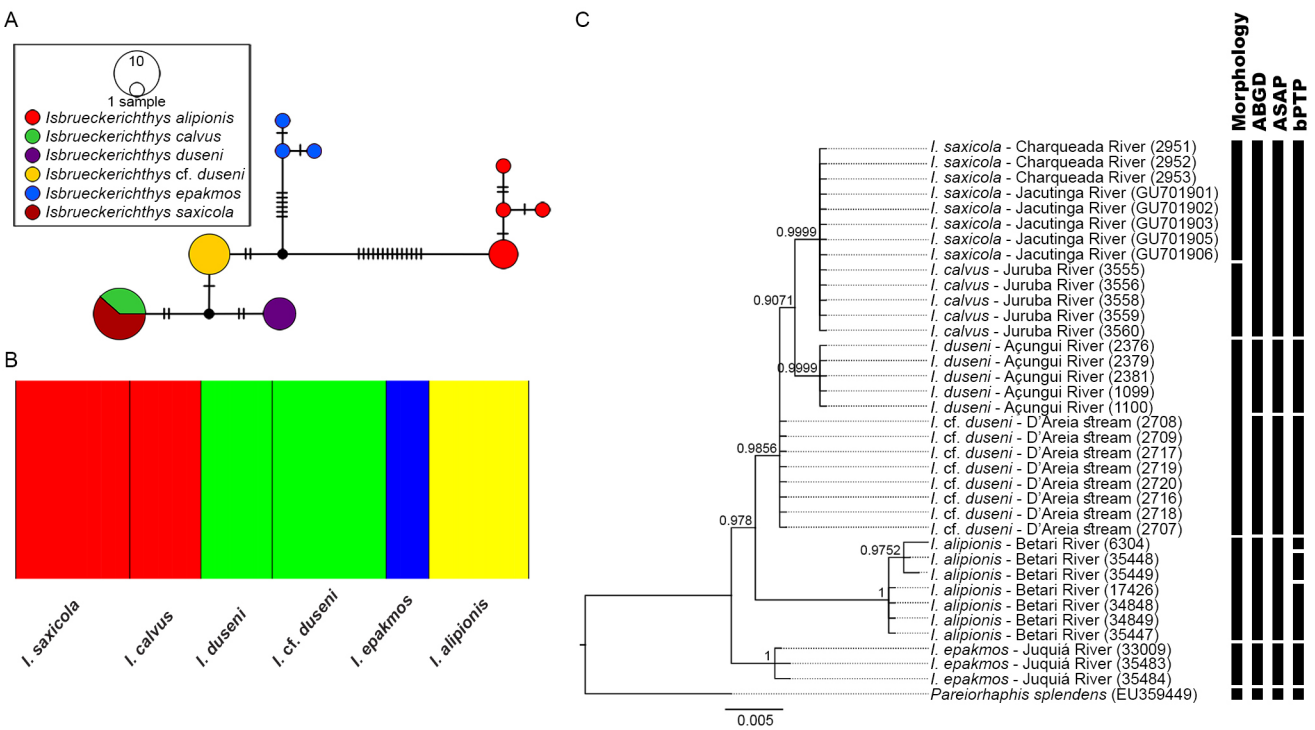

FIGURE 2 | Molecular data of *Isbrueckerichthys* specimens. **A.** Haplotype network showing the relationship among the COI sequences. **B.** Structural inference generated by BAPs (K = 4). **C.** Bayesian inference tree showing the phylogenetic relationship of *Isbrueckerichthys* sequences (numbers on the branches correspond to the posterior probability). Vertical black bars correspond to the morphological classification of the specimens, and the molecular units determined using ABGD, ASAP, and bPTP species delimitation methods.

a second cluster, and *I. epakmos* and *I. alipionis* represent independent groups (Fig. 2B). The AMOVA analysis, considering the clusters delimited by BAPs, shows a variance value of 83.98% among groups (Tab. 3), with an FST value of 0.96692 ($p < 0.05$). The pairwise FST values between each population ranged from 0.00 to 1.00 (Tab. 4).

The Bayesian phylogenetic tree revealed five supported clades: *I. epakmos*, *I. alipionis*, *Isbrueckerichthys* cf. *duseni*, *I. duseni*, and *I. calvus* + *I. saxicola* (Fig. 2C). Species delimitation methods returned different results (Fig. 2C). The ABGD method resulted in seven partitions ranging from two to nine species, with partition 4 (prior maximal distance P = 0.004642) indicating six species, including the outgroup. ABGD recognized *I. epakmos*, *I. alipionis*, *I. duseni*, and *Isbrueckerichthys* cf. *duseni* as single groups and grouped *I. calvus* and *I. saxicola*. The best partition identified by the ASAP method (ASAP-score: 3.50; *p-value*: 1.74e-02; W: 3.13e-05; Threshold dist.: 0.004094) recognized the same six species as the ABGD method. The ML solution of bPTP delimited eight species, including the outgroup. bPTP separated *I. alipionis* into three species and grouped *I. calvus* and *I. saxicola*.

TABLE 2 | Estimates of evolutionary divergence over sequence pairs among groups and standard error in the *Isbrueckerichthys* species. Bold values in main diagonal show the intraspecific genetic distance. The number of base substitutions per site from averaging over all sequence pairs between groups is shown. Analyses were conducted using the Kimura 2-parameter model. This analysis involved 36 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 612 positions in the final dataset.

	I. alipionis	*I. calvus*	*I. duseni*	*I.* cf. *duseni*	*I. epakmos*	*I. saxicola*
I. alipionis	**0.0023 (0.0012)**					
I. calvus	0.0301 (0.0072)	**0.0000 (0.0000)**				
I. duseni	0.0336 (0.0077)	0.0066 (0.0033)	**0.0000 (0.0000)**			
I. cf. *duseni*	0.0283 (0.0070)	0.0049 (0.0029)	0.0049 (0.0027)	**0.0000 (0.0000)**		
I. epakmos	0.0363 (0.0078)	0.0194 (0.0055)	0.0194 (0.0054)	0.0144 (0.0045)	**0.0022 (0.0016)**	
I. saxicola	0.0301 (0.0072)	0.0000 (0.0000)	0.0066 (0.0033)	0.0049 (0.0029)	0.0194 (0.0055)	**0.0000 (0.0000)**

TABLE 3 | Analysis of molecular variance (AMOVA) from the *Isbrueckerichthys* species. Four groups were considered: *I. alipionis*; *I. calvus* + *I. saxicola*; *I. epakmos*; *I. duseni* + *Isbrueckerichthys* cf. *duseni*. Bold values were significant ($p < 0.05$).

Source of variation	d. f.	Sum of squares	Variance components	Percentage of variation
Among groups	3	131.761	4.75474	83.98
Among populations within groups	2	9.231	0.71956	12.71
Within populations	30	5.619	0.18730	3.31
Total	35	146.611	5.66160	100
Fixation Indices **F_{SC}: 0.79346** **F_{ST}: 0.96692** **F_{CT}: 0.83982**				

TABLE 4 | Pairwise F_{ST} values among populations of *Isbrueckerichthys* (lower diagonal) and p-values (upper diagonal). Bold values were significant ($p < 0.05$).

	I. alipionis	*I. calvus*	*I. duseni*	*I.* cf. *duseni*	*I. epakmos*	*I. saxicola*
I. alipionis	–	**0.0000+-0.0000**	**0.0000+-0.0000**	**0.0000+-0.0000**	**0.0000+-0.0000**	**0.0000+-0.0000**
I. calvus	0.9523	–	**0.0180+-0.0121**	**0.0090+-0.0091**	**0.0180+-0.0121**	0.9910+-0.0030
I. duseni	0.9571	1.0000	–	**0.0090+-0.0091**	**0.0090+-0.0091**	**0.0000+-0.0000**
I. cf. *duseni*	0.9608	1.0000	1.0000	–	**0.0000+-0.0000**	**0.0000+-0.0000**
I. epakmos	0.9345	0.9615	0.9615	0.9649	–	**0.0090+-0.0091**
I. saxicola	0.9630	0.0000	1.0000	1.0000	0.9741	–

DISCUSSION

Evolutionary biologists agree that species are lineages or metapopulations that evolve separately, with disagreements remaining only about where, along the continuum of divergence, separate lineages should be considered distinct species (Wiley, 1978; Hey, 2006; Mallet, 2008). Dias Filho *et al*. (2020) argued that molecular techniques should complement classical taxonomic identification for a detailed description of organisms. Data analysis showed a restricted relationship among *Isbrueckerichthys* species, with some divergences concerning morphological identification for the samples obtained in the delimitations between the First (lowlands in Ribeira de Iguape basin) and the Second (uplands in Tibagi basin) Paraná State plateaus, Brazil.

The data obtained from COI sequences analysis demonstrated that *I. epakmos*, *I. alipionis*, and *I. duseni*, all inhabitants of the Ribeira de Iguape River basin, present K2P divergence values greater than or close to 2%, well-supported branches in the phylogenetic tree, and pairwise Fst close to 1, corroborating to the absence of gene flow and the occurrence of the nominal taxa. The Ribeira de Iguape River basin is in an intricate mountainous region with high isolating potential for aquatic organisms (Ribeiro, 2006), and Roxo *et al*. (2012) proposed that the ancestor of *Isbrueckerichthys* inhabited tributaries from this basin at least 20 million years ago. Langeani (1990) stated that most Neoplecostomini (Neoplecostominae in that work) species have low vagility and difficulties adapting to streams that occur at low altitudes due to the low oxygen concentration in the water compared to rivers in higher regions. Thus, streams in valleys between mountains (low altitude) could hinder the dispersal of Neoplecostomini species into different tributaries of the Ribeira de Iguape River basin (Fig. 1). These geological and ecological factors and evolutionary times allow for congruence between the molecular and morphological divergence data for *I. epakmos*, *I. alipionis*, and *I. duseni*.

The methods for species delimitation included *I.* cf. *duseni* (D'Areia stream), sampled in the border of the Ribeira de Iguape basin, the limit with Tibagi basin in Paraná State, as a new taxon for Ribeira de Iguape basin. Comparing specifically *I. duseni vs. I.* cf. *duseni*, 0.49% of K2P genetic distance was observed. However, the pairwise Fst showed gene flow restriction, and branches well-supported in the phylogenetic tree topology for *I. duseni vs. I.* cf. *duseni* were shown. Some Neotropical fish species demonstrate distinct

taxonomic entities with genetic distances lower than 2%, although they are arranged in monophyletic groups (Bellafronte *et al*., 2013; Traldi *et al*., 2020). Based on molecular data, several studies have highlighted cryptic diversity for neotropical freshwater fish (Melo *et al*., 2016; Guimarães *et al*., 2019; Mateussi *et al*., 2019; Souza *et al*., 2021, 2023; Cardoso *et al*., 2023). In addition, as mentioned above, the specimens of *Isbrueckerichthys* could have gene flow restriction due to their biological features associated with the geological formation of the Ribeira de Iguape tributaries. There is a valley between the sample localities of *I. duseni* and *I.* cf. *duseni* (Fig. 1), which, according to Langeani (1990), makes dispersal difficult and, in this case, could contribute to genetic isolation. The data suggests that a molecular operational taxonomic unit for *I.* cf. *duseni* in the Ribeira de Iguape basin or a case of incipient speciation should be considered.

The genetic comparison among *I. duseni*, *I.* cf. *duseni*, *I. saxicola*, and *I. calvus* also demonstrated low K2P genetic distance (0.49–0.66%), high Fst, and branches well-supported in the phylogenetic tree. Despite low K2P values, *I. duseni* and *Isbrueckerichthys* cf. *duseni* are geographically isolated from *I. saxicola* and *I. calvus* by the Ponta Grossa Arch, an uplift of the relief that isolated the Ribeira de Iguape basin from upland rivers (Fig. 1), such as Tibagi basin (Ribeiro, 2006). *Isbrueckerichthys duseni* and *I.* cf. *duseni* were considered valid taxa in species delimitation methods, but a morphological and molecular data incongruence was observed between *I. saxicola* and *I. calvus*. These two species were described from the Tibagi basin by Jerep *et al*. (2006) having morphological differences with congeners including: bicuspid teeth, hypertrophied odontodes, and a more extended spine in the pectoral fin. The morphological differentiation between *I. calvus* and *I. saxicola* is based on the number of odontodes on the abdominal platelet (six or more in *I. saxicola vs.* up to six in *I. calvus*), by the absence/presence of a flattened area in the inferior portion of the two first plates of the lateral line, by the degree of exposition of the cleithrum and by the degree of convexity and exposition of the supraoccipital plane (Jerep *et al*., 2006). Although the morphological description of Jerep *et al*. (2006) is well-detailed, the morphological divergence is not accompanied by a genetic divergence. All methods based on the molecular species delimitation used in this study, using a single locus analysis, agree with *I. saxicola* and *I. calvus* as a single taxon. In addition, a pairwise Fst 0 was estimated between the localities of the *I. saxicola* and *I. calvus* samples, indicating no population structure.

In turn, it has been proposed that recent speciation in newly inhabited areas may present an intermediary stage of speciation due to the short space of evolutionary time for diversification, especially cases of incomplete lineage sorting and lack of divergence (Roxo *et al*., 2012, 2014, 2017). Neoplecostomini species occurring in the uplands of the Tibagi basin, like the case of *Isbrueckerichthys*, were proposed to inhabit this new area from dispersion of fishes through headwaters capture events between the coastal regions and those more elevated in the borders of the crystalline shield (Ribeiro, 2006; Roxo *et al*., 2012, 2014; Dagosta *et al*., 2024). Roxo *et al*. (2012) also suggested that the dispersal occurred around 4.8 (2.3–7.8), 5.9 (2.9–9.5), and 10.6 (5.5–16.5) million years ago. Based on this information, it is possible to verify that the morphological diversification between *I. calvus* and *I. saxicola* is not accompanied by a rate of nucleotide substitution, probably due to the short time of dispersal and distribution of *Isbrueckerichthys* in the Tibagi River basin.

Even with the emergence of combined multiple data sets strategies applied to diversity analysis, according to some authors, it is a challenge to evaluate species delimitation in considering demes that comprise geographically widespread series of similar individuals (de Queiroz, 2007; Willis, 2017), or those that emerged from a short period of dispersal in evolutionary diversification. However, also is expected a degree of phenotypic (morphological, ecological, and behavioral) divergence among sub-populations due to genetic drift and local adaptation (López-Fernández *et al*., 2005; de Queiroz, 2007; Zapata, Jiménez, 2011; Willis *et al*., 2017). At this point, the morphological differences between *I. calvus* and *I. saxicola* could be explained by their dispersal in distinct tributaries (environments) in the Tibagi basin. To solve these cases of incomplete lineage sorting and lack of divergence, as here demonstrated in *Isbrueckerichthys*, some authors argue that a multilocus analysis, phylogenomics methods, and multiple datasets are important for both phylogenetic resolution and in-depth investigation of speciation (López-Fernández *et al*., 2005, 2010; Ilves *et al*., 2018).

Integrative studies of molecular and morphological investigations may be considered for species delimitation because they represent complementary approaches practical for the group's phylogeny, systematics, evolution, and phylogeography. However, groups with recent diversification (as revealed by similar or identical genetics coupled with morphological differentiation) still challenge integrative taxonomy. Here, there is low genetic divergence among *Isbrueckerichthys* that inhabit the border of Ribeira de Iguape basin in Paraná State and those from uplands in the Tibagi basin, which can be considered recent speciation cases. In *I. calvus* and *I. saxicola* in the Tibagi basin, molecular analysis indicates that morphological diversification between the species is not accompanied by genetic diversification.

ACKNOWLEDGMENTS

MRV received financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 313566/2023–2) and Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Estado do Paraná (009–2016). RBA and MA received financial support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES - Finance Code 001). CO received financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grant 2020/13433–6) and CNPq (processes 306054/2006–0 and 441128/2020–3).

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Neotropical Ichthyology

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Official Journal of the
Sociedade Brasileira de Ictiologia

ETHICAL STATEMENT

Fishes were collected with authorization of the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO), Sistema de Autorização e Informação em Biodiversidade (SISBIO - License Ids 13843–6 and 15117–4). This study procedures agree with the Ethics Committee of Animal Usage of the Universidade Estadual de Ponta Grossa (Protocol: 06/2019).

COMPETING INTERESTS

The author declares no competing interests.

HOW TO CITE THIS ARTICLE

- **Almeida RB, Azambuja M, Nogaroto V, Oliveira C, Roxo FF, Zawadzki CH, Vicari MR.** DNA barcode shows discordant cases among morphological and molecular species identification in *Isbrueckerichthys* (Siluriformes: Loricariidae). Neotrop Ichthyol. 2024; 22(3):e240040. https://doi.org/10.1590/1982-0224-2024-0040