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ORIGINAL ARTICLE

Integrated analysis reveals a new species of *Corydoras* Lacépède, 1803 (Siluriformes: Callichthyidae) in the lower Iguassu River, Brazil

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Abstract

Corydoras is the richest genus of Corydoradinae, and many of its species have not been identified to date. We characterized *Corydoras carlae* and *Corydoras* sp. by performing cytogenetic, morphometric, and molecular analyses to facilitate correct identification and species delimitation and contribute an understanding of the evolutionary process of this group of fish. Individuals of *C. carlae* were collected in the Florido River, a tributary of the Iguassu River upstream of Iguassu Falls, and individuals of *Corydoras* sp. were collected in the Poço Preto River, a tributary of the Iguassu River downstream of Iguassu Falls. *Corydoras* sp. presented an extra rDNA 5S marker in an interstitial position on the short arm of one of the chromosomes of the submetacentric pair 15. Mitochondrial (COI) and nuclear (RAG1) sequences were efficient in discriminating *C. carlae* and *Corydoras* sp. Both species had exclusive haplotypes, which suggests the absence of gene flow between species. Furthermore, species delimitation analysis (GMYC and ABGD) suggested two MOTUs for *Corydoras* specimens from the Iguassu River. Differences in morphometric proportions were also observed. Considering the data gathered in this study, *C. carlae* and *Corydoras* sp. comprise distinct evolutionary lineages that are probably undergoing a recent speciation process.

Keywords Fish cytogenetic markers · Ribosomal DNA · Morphometric proportions · DNA barcoding

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Introduction

Corydoras is the richest genus of Corydoradinae and, consequently, of Callichthyidae and of Siluriformes, currently comprising 175 valid species (Tencatt et al., 2019; Lima & Britto, 2020; Fricke et al., 2021) distributed in the main rivers of South America. Although comprehensive studies of group systematics have published (e.g., Alexandrou et al., 2011; Britto, 2003; Eigenmann & Eigenmann, 1890; Ellis, 1913; Gosline, 1940; Nijssen, 1970; Nijssen & Isbrücker, 1967, 1980, 1983, 1986), knowledge about the taxonomy of many species and their phylogenetic relationships remains incipient (Tencatt & Ohara, 2016).

An unpublished taxonomic review of *Corydoras paleatus* (Jenyns, 1842) revealed the presence of four new species previously attributed to *C. paleatus*: *Corydoras* sp. A, *Corydoras* sp. B, *Corydoras* sp. C, and *Corydoras* sp. D (Tencatt, 2013). Subsequently, Tencatt et al. (2016), also in a review of *C. paleatus*, attributed to *C. longipinnis*, although not explicitly, the species previously identified as *Corydoras* sp. A, while *Corydoras* sp. C was described as *C. froehlichi*. *Corydoras* sp. B and *Corydoras* sp. D were not included by the authors in this review. For *Corydoras* sp. D, the scarcity of biological material (only five individuals) was the main limiting factor for its formal description, since the authors were able to clearly delimit this species morphologically (see Tencatt, 2013). In contrast, *Corydoras* sp. B had abundant material but could not be clearly diagnosed from its sympatric *Corydoras carlae* Nijssen & Isbrücker, 1983 (Tencatt personal observation). In fact, *C. carlae* and *Corydoras* sp. B present similar color and morphology patterns, having been diagnosed by Tencatt (2013) only by differences in the size of their dorsal and pectoral spines (dorsal spine 13.7–22.5% in standard length; pectoral spine 15.1–22.4% in standard length in *Corydoras* sp. B vs. 26.6–33.6 and 25.9–31.9 in *C. carlae*). Both species are apparently restricted to the Iguassu River basin (Ingenito et al., 2004; Rocha et al., 2016). *Corydoras carlae* was recorded upstream from Iguassu Falls, while *Corydoras* sp. B (hereinafter referred to as *Corydoras* sp.) was found only in the Poço Preto Stream, a tributary of the lower Iguassu River, downstream of Iguassu Falls and located in Iguassu National Park.

To contribute to knowledge regarding the diversity of species of *Corydoras*, different methods can be used for the correct delimitation of species. Most cytogenetic studies in *Corydoras* are restricted to conventional analysis and have demonstrated the existence of different diploid numbers, which can vary from 2n = 40 chromosomes in *C. nattereri* to 2n = 134 chromosomes in *C. aeneus* (Oliveira et al., 1990, 1992, 1993). These results suggest an intense polyploidy process in the diversification and evolutionary history of *Corydoras* (Oliveira et al., 1988, 1993; Turner et al., 1992). The distribution pattern of heterochromatin, as well as the location and quantity of chromosomes carrying Ag-NORs, is highly variable cytogenetic characteristics in the genus. However, little is known about the locations of the different types of rDNA. Thus, solving this gap in genetic knowledge is essential to better understand the relationships between species of *Corydoras* (Almeida et al., 2013; Artoni et al., 2006; Pazza et al., 2005).

Morphometry, similar to cytogenetics, is a tool that can also help elucidate systematic relationships within a group, providing precise interpretation and comparison of the variation patterns of quantitative characters (Blackith & Reyment, 1971; Cavalcanti & Lopes, 1990). This technique has been used to highlight differences in body shape in relation to fish size, which allows relationships between individuals to be detected and interpreted (Bemvenuti & Rodrigues, 2002; Shibatta & Hoffman, 2005; Almeida et al., 2012). In this context, fish populations isolated in headwater streams may present morphological divergences as a result of a change in gene frequency, leading to speciation through reproductive incompatibility (Castro, 1999).

Molecular techniques have strengthened the study of fish systematics in recent years, including *Corydoras* and other Callichthyidae genera (e.g., Shimabukuro-Dias et al., 2004). The development of molecular tools and methods for delimiting species makes it possible to more precisely estimate the existing biodiversity (Camargo & Sites, 2013; Pinacho-Pinacho et al., 2018). Thus, the use of DNA nucleotide sequences can help in the correct identification of species, especially in cases where real biodiversity cannot be detected by traditional taxonomy and systematic methods based on morphology (Bickford et al., 2007; Larson et al., 2016). In addition, the genetic characterization of *C. carlae* and *Corydoras* sp. can reveal important information about genus diversity. Analyses of different molecular markers of mitochondrial and nuclear DNA with different evolutionary rates are important for obtaining a better understanding of the evolutionary process in neotropical fishes (Fabrin et al., 2014).

Thus, in view of the morphological complexity of *Corydoras* fish species, the present study attempted to characterize *Corydoras carlae* and *Corydoras* sp. of the lower Iguassu River basin through cytogenetic, morphometric, and molecular analyses to help in the correct identification and delimitation of species in addition to contributing to an understanding of the evolutionary process of this group of fish.

Methods

Study area and sampling

Seventeen *Corydoras carlae* specimens (Fig. 1a) were collected from the Florido River (26°00′04″S; 53°37′32″W;

Fig. 2), a tributary of the Capanema River, which flows into the Iguassu River upstream of Iguassu Falls. In addition, 24 *Corydoras* sp. specimens (Fig. 1b) were collected at two sites (site 1: 25°36′45.5″S; 54°25′50.7″W and site 2: 25°37′19.7″S; 54°26′53.1″W; Fig. 2) in the Poço Preto Stream, a tributary that flows into the Iguassu River downstream from Iguassu Falls. Voucher specimens were deposited in the fish collection of the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (NUPELIA), Universidade Estadual de Maringá, Paraná, Brazil, as *C. carlae* (NUP 17885) and *Corydoras* sp. (NUP 14261 and NUP 17887).

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, approved by the Committee on the Ethics of Animal Experiments of the Universidade Estadual do Oeste do Paraná (License Number: Protocol 13/09 – CEUA/Unioeste). Fish were collected with permission of IAP (Instituto Ambiental do Paraná, permit n° 43966/2015). Before the evisceration process, the individuals were euthanized by an overdose of clove oil (Griffiths, 2000).

Cytogenetic analysis

Cytogenetic analyses were performed on 10 individuals of *C. carlae* (four females and six males) and 15 individuals of *Corydoras* sp. (eight females and seven males). To obtain metaphasic chromosomes from cells extracted from the kidney, the methodology described by Bertollo et al. (1978) was used. The nucleolar organizer regions (NORs) were detected by means of silver nitrate staining (Ag-NORs), according to Howell and Black (1980), and analysis of C-positive heterochromatin (C-bands) followed the basic procedure of Sumner (1972), with some minor adaptations (Lui et al., 2012). Physical mapping of 5S rDNA and 18S rDNA was carried out by fluorescence in situ hybridization (FISH) according to Pinkel et al. (1986) and modifications suggested by Margarido and Moreira-Filho (2008) using DNA probes obtained from *Megaleporinus obtusidens* (cited as *Leporinus elongates* by Martins & Galetti, 1999) and *Prochilodus argenteus* (Hatanaka & Galetti, 2004), respectively. Probes were labeled by the nick translation method with digoxigenin-11-dUTP (5S rDNA) and biotin-16-dUTP (18S rDNA) (Roche®). Detection of signals was performed with antidigoxigenin-rhodamine (Roche®) as a probe for 5S rDNA and amplified avidin-FITC with biotinylated anti-avidin (Sigma-Aldrich) as a probe for 18S rDNA, with the chromosomes counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 50 µg/mL).

The slides were analyzed under an optical microscope, and chromosomal counts and more detailed observations of the metaphases were made with a 100× objective. The best metaphases were captured with a DP 71 digital camera coupled to the BX 61 epifluorescence microscope using DP Controller software, version 3.2.1.276. After capturing the images, the chromosomes were classified as metacentric (m), submetacentric (sm), and subtelocentric (st) according to their arm ratio (Levan et al., 1964). For determination of the fundamental number (FN) or number of chromosome arms, the m, sm, and st chromosomes were considered to

Fig. 1 Specimens of *Corydoras carlae*, the holotype (IRSBN 688, 41.8 mm SL) (**a**) and *Corydoras* sp. (40.6mm SL; voucher number NUP 17887) sampled in the Poço Preto Stream (**b**), both from the Iguassu River basin

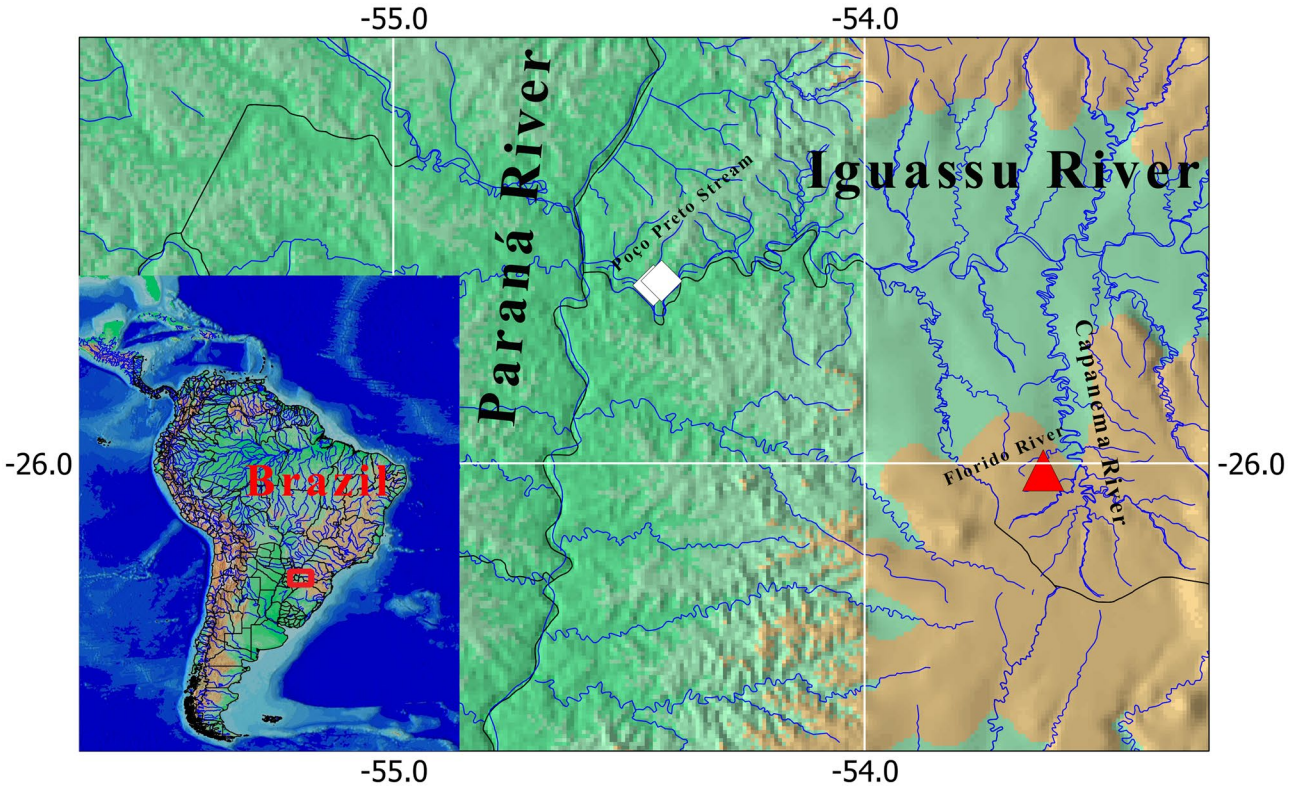


Fig. 2 Sampling sites in the Iguassu River basin, where individuals from *Corydoras carlae* (red triangle, Florido River) and *Corydoras* sp. (white lozenges, Poço Preto Stream) were collected

bear two arms, and the acrocentric chromosomes were considered to bear only one arm.

Morphometric analysis

Morphometric character measurements of 17 individuals of *C. carlae* and 24 individuals of *Corydoras* sp. were performed using a digital caliper. The measurements were obtained according to Reis (1998) with some additions, which are all listed hereafter: standard length, thorax length, abdomen length, body height at the origin of the dorsal fin, predorsal distance, prepelvic distance, preanal distance, preadipose distance, dorsal-fin spine length, pectoral-fin spine length, caudal peduncle height, adipose-fin spine length, distance between the end of the base of the dorsal fin and the origin of the spine of the adipose fin, dorsal-fin base length, anal-fin base length, maximum width of the cleiter, head length, maxillary barbell length, head height, interorbital distance, horizontal orbit diameter, snout length, and internareal distance. To navigate the effect of the size of the measured specimens, proportions were calculated using the measurement of each variable (mm) in relation to the standard length (for measurements referring to the post cephalic portion of the body) and in relation to the head length (measurements referring to the head).

To summarize the matrix of morphometric variables, we applied a principal component analysis (PCA) using PC-ORD 5.0 software (McCune & Mefford, 2007). To determine which principal components would be retained for interpretation, we used the broken-stick model as the criterion (Jackson, 1993). To test the null hypothesis that neither species showed morphological differences, PERMANOVA multivariate permutational variance analysis was used with the Bray–Curtis index obtained with 999 random permutations (Anderson, 2001). The level of significance adopted was $p < 0.05$.

Molecular analysis

The DNA of 22 *Corydoras* specimens was isolated using the Wizard® Genomic DNA Purification Kit (Promega), stored at – 20 °C, and quantified by electrophoresis on a 1% agarose gel compared to a 100 bp ladder molecular standard (Ludwig). Mitochondrial and nuclear DNA fragments were amplified via polymerase chain reaction (PCR). A partial region of the mitochondrial cytochrome c oxidase subunit I (COI) gene of approximately 700 base pairs (bp) was amplified with the primers Fish_F1 (5′ – TCA ACC AAC CAC AAA GAC ATT GGC AC – 3′) and Fish_R1 (5′ – TAG ACT TCT GGG TGG CCA AAG AAT CA – 3′) (Ward et al., 2005). For

amplification of the nuclear gene recombination activating protein 1 (RAG1), the primer pair RAG1 F (5′ – AAG GAG AGG GGT ATA GAT GAT A – 3′) and RAG1 R (5′ – GCA AAA CGC TGA GAG TTG AA – 3′) (Alexandrou et al., 2011) was used, which resulted in a fragment of approximately 1,000 bp.

Mitochondrial and nuclear fragments were amplified in independent PCRs. The reaction mixture consisted of Tris-KCl buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 1.5 to 2 mM $MgCl_2$, 0.6 µM of each primer, 0.4 mM of each dNTP, 3 U of Taq DNA polymerase (Invitrogen), 25 ng of DNA and filtered/deionized water (Milli-Q) for a final volume of 25 µL. The amplifications of the COI gene occurred in a thermocycler programmed for the following thermal profile: an initial cycle of 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 45 s at 58 °C, and 1 min at 72 °C, with an additional last step of 5 min at 72 °C. For amplification of the RAG1 fragment, an initial cycle of 4 min at 94 °C was used, followed by 40 cycles of 1 min at 94 °C, 45 s at 59 °C and 90 s at 72 °C, in addition to a last step of 7 min at 72 °C. The amplification efficiency was confirmed by electrophoresis on a 1% agarose gel and staining with SYBR Safe (Invitrogen).

Subsequently, the amplified samples were purified with a PureLink® PCR Purification Kit (Invitrogen). After purification, COI and RAG1 fragments were used in sequencing reactions, with the primers Fish_F1 and RAG1 F, respectively, using the BigDye® Terminator v3.1 Cycle Sequencing kit, in automatic sequencer ABI 3500 DNA Analyzer (Life Technologies), according to manufacturer's instructions.

The nucleotide sequences obtained were edited using BioEdit software (Hall, 1999) and aligned with Clustal Omega software (Sievers et al., 2011). The genetic distance and the frequencies of nucleotide bases were estimated using MEGA X software (Kumar et al., 2018). COI and RAG1 sequences from other species of *Corydoras* available in BOLD Systems and GenBank were included in the analyses; *Aspidoras* sp. was used as an outgroup (Table S1).

The selection of the best-fit model of nucleotide evolution and the partitions were conducted using PartitionFinder 2.1 software (Lanfear et al., 2012). Maximum likelihood trees were reconstructed with raxmlGUI software (Silvestro & Michalak, 2012) using the partitions established by PartitionFinder (1st, 2nd, and 3rd bases, for COI; and 1st and 2nd codon, and 3rd codon RAG1) and the GTR + G model. A rapid bootstrap algorithm and autoMRE function for resamplings were implemented.

Bayesian ultrametric tree reconstructions were performed with BEAST 1.8.2 software with an input file generated in BEAUti 1.8.0 (Drummond et al., 2012); the birth–death process of speciation was used as a tree prior, and a strict molecular clock was used for both COI and RAG1. The COI region was partitioned according to codon bases (1st, 2nd, and 3rd bases) using the TN93 substitution model, whereas RAG1 was partitioned according to codon bases (1st and 2nd bases and 3rd bases) using the HKY model. Analysis ran for 20,000,000 (for COI) and 10,000,000 (for RAG1) generations with a sample frequency of 1,000. The final trees were calculated after 20% burn-in. The length of burn-in was determined by examining traces in Tracer 1.6 (Rambaut et al., 2014), considering > 200 as an appropriate effective sample size (ESS) value. Support for nodes was determined using posterior probabilities.

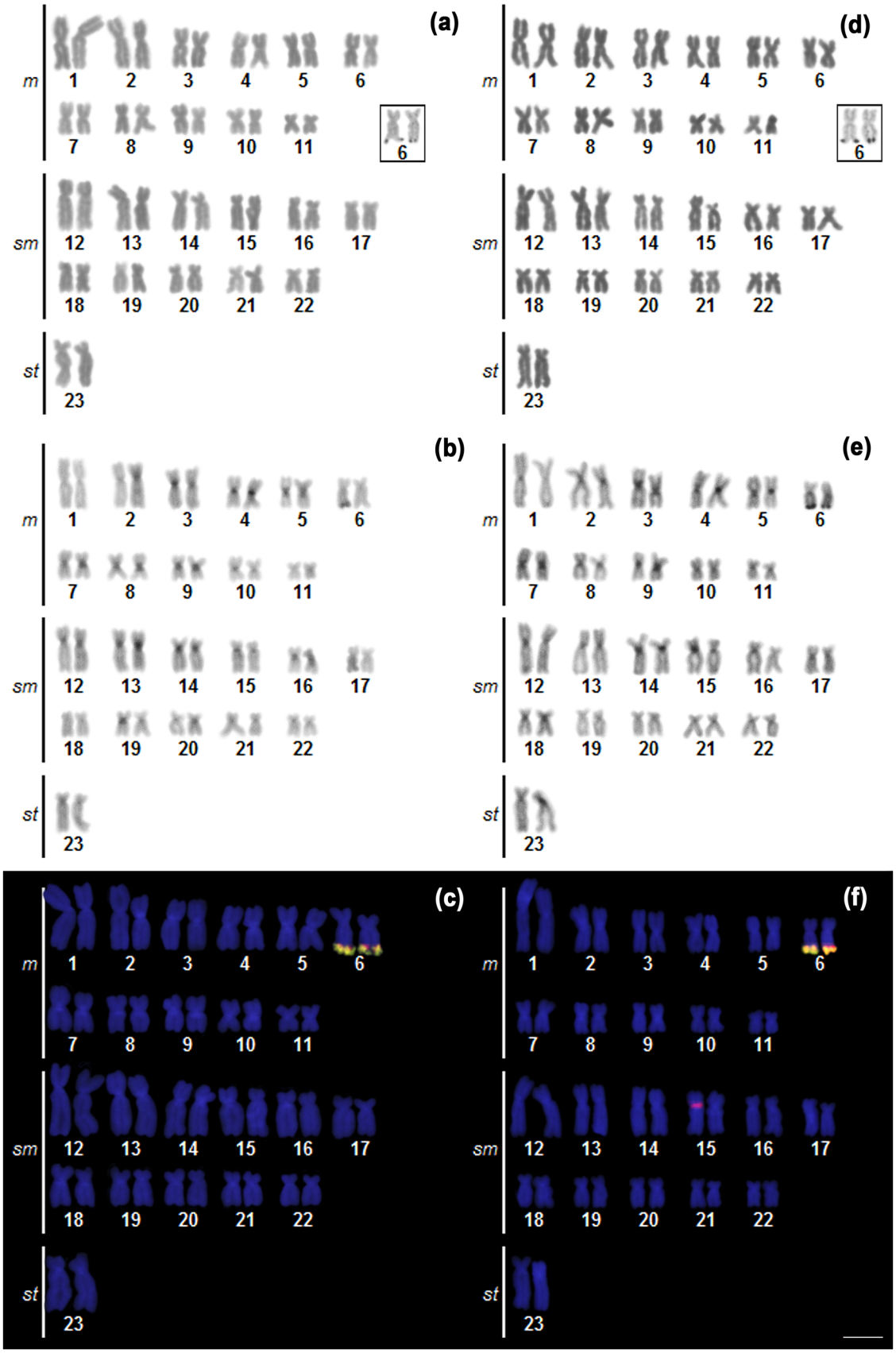
To estimate the time of divergence between *C. carlae* and *Corydoras* sp., an outgroup-rooted phylogenetic tree was built based on the sequences of the COI gene, and with the assumption of a calibrated molecular clock, which admits a constant mutation rate over time. The assumed calibrated molecular clock attributed an uncorrected mutation rate of 1.2% per million years (Mya) to the COI gene. This is an average mutation rate since geological and fossil data show a mutation rate in fish ranging from 1.1 to 1.3% per Mya (Bermingham et al., 1997; Near et al., 2003). For the construction of the tree, the same procedure described above was used for the ultrametric tree. Inferences of clade ages are presented as 95% highest posterior density (HPD).

Additionally, specifically for the COI gene, to identify molecular operational taxonomic units (MOTUs) (Hebert et al., 2003), methods for species delimitation were implemented to identify the specific boundaries in *C. carlae* and *Corydoras* sp. The Bayesian ultrametric tree was used for the general mixed Yule coalescent method (GMYC; Pons et al., 2006) using R Studio software (R Development Core Team, 2020) and the splits package (Fujisawa & Barraclough, 2013). The maximum likelihood gene tree was used for the Poisson tree process model (PTP; Zhang et al., 2013) delimitation test, which was performed online (http://species.h-its.org). The ABGD method was conducted on the online server http://wwwabi.snv.jussieu.fr/public/abgd using the default parameters and Kimura (K80) model of nucleotide substitution.

Results

Cytogenetic data

The diploid number, karyotype formula, and FN were the same for *Corydoras carlae* and *Corydoras* sp., with 2n = 46 chromosomes, composed of 22 metacentric chromosomes, 22 submetacentric chromosomes, and two subtelocentric chromosomes for both sexes and FN equal to 92 (Fig. 3a-d). Silver nitrate impregnation identified Ag-NOR in a terminal position in the long arm of metacentric pair six in the two species (Box Fig. 3a, d). FISH with 18S rDNA coincided with the marking of silver nitrate in *C. carlae* and *Corydoras*


(a)
m 1 2 3 4 5 6
7 8 9 10 11
6
sm 12 13 14 15 16 17
18 19 20 21 22
st 23
(d)
m 1 2 3 4 5 6
7 8 9 10 11
6
sm 12 13 14 15 16 17
18 19 20 21 22
st 23
(b)
m 1 2 3 4 5 6
7 8 9 10 11
sm 12 13 14 15 16 17
18 19 20 21 22
st 23
(e)
m 1 2 3 4 5 6
7 8 9 10 11
sm 12 13 14 15 16 17
18 19 20 21 22
st 23
(c)
m 1 2 3 4 5 6
7 8 9 10 11
sm 12 13 14 15 16 17
18 19 20 21 22
st 23
(f)
m 1 2 3 4 5 6
7 8 9 10 11
sm 12 13 14 15 16 17
18 19 20 21 22
st 23

◂**Fig. 3** Karyotypes of *Corydoras carlae* (left column) and *Corydoras* sp. (right column) stained with Giemsa (**a**; **d**), C-banded (**b**; **e**) and double FISH with 5S rDNA (red) and 18S rDNA (green) (**c**; **f**) probes. The boxes contain the pairs carrying the Ag-NORs. The bar represents 10 µm

sp., featuring a simple NOR system (Fig. 3c, f). In addition, 5S rDNA cistrons colocalized with 18S rDNA were observed for *C. carlae* and *Corydoras* sp. (Fig. 3c, f and Fig. 4). However, *Corydoras* sp. presented an extra marker of 5S rDNA located in an pericentromeric position on the short arm of one of the chromosomes of submetacentric pair 15 (Fig. 3f).

Heterochromatins were observed in the centromeric/pericentromeric region of most of chromosomes of the complement, in addition to being associated with NORs in both species (Fig. 3b, e).

Morphometric data

The proportions analysis calculated from the morphometric measurements of *C. carlae* and *Corydoras* sp. demonstrated differences in body height/standard length, average of 29.1 mm in *C. carlae* and 34.2 mm in *Corydoras* sp., interorbital distance/head length, average of 52.6 mm in *C. carlae* and 39.3 mm in *Corydoras* sp. and the horizontal diameter of the orbit/head length, average of 30.0 mm in *C. carlae* and 23.4 mm in *Corydoras* sp. (Table 1).

The first two axes of the principal component analysis showed eigenvalues greater than the eigenvalues of the broken-stick and were retained for interpretation. These two axes showed an accumulated explained variance of 35% (Table 2). The results of PERMANOVA indicated that *C. carlae* and *Corydoras* sp. showed significant differences in morphology (*Pseudo*-F = 1.98; $p < 0.01$). Thus, *Corydoras* sp. have a body height in relation to the standard length greater than that found for *C. carlae*. On the other hand, the interorbital distance and the horizontal diameter of the orbit in relation to the head length are greater in *C. carlae* than in *Corydoras* sp. (Fig. 5).

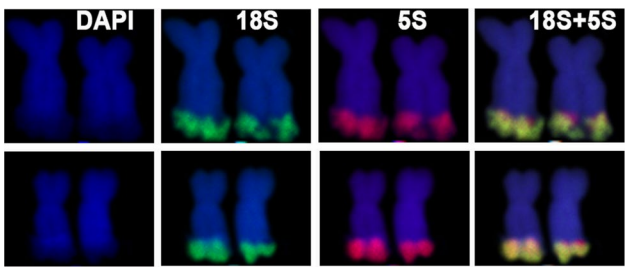


Fig. 4 Chromosome pair 6 bearing 5S (red) and 18S (green) rDNA showing the synteny of these sites. In the first line is *C. carlae* and in the second line is *Corydoras* sp

Molecular data

A total of 76 sequences of the *Corydoras* mitochondrial COI gene were used in this study, including 22 sequences of 634 bp (base pair) of specimens collected in the Iguassu River basin (*C. carlae* and *Corydoras* sp.) and 54 COI sequences of *Corydoras* species obtained from GenBank (Table S1). For the nuclear RAG1 gene, 51 nucleotide sequences were obtained, 20 sequences of 839 bp referring to the Iguassu River specimens, and 31 *Corydoras* RAG1 sequences obtained from BOLD and GenBank (Table S1). All sequences generated in this study were deposited in GenBank (GenBank accession numbers = MT846090—MT846111 for COI sequences; MT855475—MT855494 for RAG1 sequences). The nucleotide composition of the COI fragment for specimens of *Corydoras* sp. and *C. carlae* was 27.7% (T), 28.8% (C), 26.2% (A), and 17.3% (G), while for RAG1, it was 25.1% (T), 20.8% (C), 27.9% (A), and 26.2% (G). Polymorphic and species-specific nucleotide sites are described in Table S2. A single mitochondrial haplotype COI was identified among the specimens of *C. carlae*, and there were two haplotypes among *Corydoras* sp. For the RAG1 fragment, six haplotypes were observed in *C. carlae* and three in *Corydoras* sp. No shared haplotypes were observed between *C. carlae* and *Corydoras* sp. In some regions of the nuclear sequence of RAG1, mainly in specimens of *C. carlae*, sites were observed in heterozygous states, which were identified as double strong peaks of the same height or very close heights seen in the chromatograms.

Mitochondrial (COI) and nuclear (RAG1) nucleotide sequences were efficient in discriminating *Corydoras* species, revealing high values of posterior probabilities or bootstraps supporting the clades in both dendrograms (Figs. 6 and 7). *Corydoras carlae* and *Corydoras* sp. from the Iguassu River basin were grouped into two distinct clades in both analyses. The clustering of the specimens was performed according to their morphological identification. Some species of *Corydoras*, such as *C. nattereri*, *C. paleatus*, and *C. aeneus*, according to data regarding the COI sequences, and *C. diphyes* and *C. ehrhardti*, for RAG1, formed more than one clade, and some were even non-monophyletic. One of the clades formed by specimens of *C. diphyes* was allocated within the larger clade constituted by the species of *Corydoras* from the Iguassu River, suggesting nonmonophyletic conditions for *C. carlae* and *Corydoras* sp.

According to the results of divergence time estimation, the approximate origin of the species of *Corydoras* evaluated here was 22.1 Mya (95% HPD 26.1-18.3 Mya) (Fig. 6). The clade formed by *Corydoras* sp., *C. carlae*, *C. nattereri*, *C. paleatus*, *C. ehrhardti*, and *C. sterbai* was estimated to have originated 8.7 Mya (95% HPD 10.8-6.7), in the early Miocene. The event that originated the clade of *Corydoras* sp. and *C. carlae* occurred more

Table 1 Morphometric measurements of the specimens of *Corydoras carlae* samples in the Florido River and *Corydoras* sp. sampled in the Poço Preto Stream, lower Iguassu River basin, and morphometric proportions in relation to the standard length (SL) and the head length (HL)

	Corydoras carlae		*Corydoras* sp.	
	Minimum–maximum	**Average**	**Minimum–maximum**	**Average**
Standard length (mm)	21.5–38.4	29.5	29.2–41.9	35.55
% standard length				
Thorax length	12.4–24.8	18.6	17.4–25.5	21.4
Abdomen length	28.5–37.8	33.1	31.2–41.0	36.1
Body height at the origin of the dorsal-fin	23.3–35.0	29.15	31.5–37.0	34.2
Predorsal distance	42.3–59.3	50.8	44.1–65.3	54.7
Prepelvic distance	38.0–54.0	46.0	40.5–46.2	43.3
Preanal distance	66.3–80.3	73.3	63.0–99.7	81.3
Preadipose distance	57.7–88.1	72.9	81.0–109.6	95.3
Dorsal-fin spine length	19.6–30.9	25.2	16.9–23.8	20.3
Pectoral-fin spine length	15.7–27.0	21.3	19.3–27.3	23.3
Caudal peduncle height	12.2–14.8	13.5	12.8–17.3	15.0
Adipose-fin spine length/standard length	6.2–10.9	8.5	6.8–11.4	9.1
Distance between the end of the base of the dorsal-fin and the origin of the spine of the adipose-fin/standard length	14.6–33.6	24.1	17.9–24.8	21.3
Dorsal-fin base length/standard length	12.4–22.0	17.2	15.5–20.6	18.5
Anal-fin base length/standard length	6.2–12.2	9.2	6.0–11.4	8.7
Maximum width of the cleiter/standard length	8.0–14.0	11.0	9.5–17.7	13.6
Head length (mm)	5.1–10.2	7.65	7.7–12.4	10.0
% head length				
Maxillary barbell length	30.3–70.5	50.4	27.7–65.9	46.8
Head height	73.5–115.2	94.3	79.8–107.7	93.7
Interorbital distance	32.8–72.5	52.6	33.3–45.4	39.35
Horizontal orbit diameter	16.9–43.1	30.0	19.4–27.5	23.45
Snout length	41.3–78.4	59.8	39.2–58.4	48.8
Internareal distance	12.3–35.2	23.7	14.5–30.3	22.4

recently; in the Pleistocene, 1.1 Mya (95% HPD 1.7-0.5 Mya). The average values of genetic distance ranged from 3.8% (between *C. nattereri* and *C. ehrhardti*) to 18.4% (between *C. sterbai* and *C. flaveolus*) for COI sequences and from 0.7% (between *C. ehrhardti* and *C. natttereri*) to 4.3% (between *C. difluviatilis* and *C. sterbai*) for RAG1. The average genetic distance between *C. carlae* and *Corydoras* sp. was 1.1% for COI and 0.5% for RAG1 (Table 3).

The ultrametric Bayesian tree was subjected to the GMYC delimitation method, and 19 MOTUs were obtained. Using the PTP method, based on the maximum likelihood tree, 17 MOTUs were delimited, while according to the ABGD method, 16 MOTUs were defined (Fig. 6). The difference in the number of MOTUs delimited by the three methods was related to *Corydoras* sp., *C. carlae*, *C. paleatus*, *C. ehrhardti*, and *C. aeneus*. Regarding the species in the Iguassu River basin, two MOTUs were delimited by the GMYC and ABGD methods, with each MOTU referring to a species (*Corydoras* sp. and *C. carlae*), while a single MOTU was defined by the PTP method (Fig. 6).

Discussion

Cytogenetics

Our cytogenetic analyses revealed that the two *Corydoras* species studied shared the same diploid number (2n = 46) and karyotype formula, including both species within group 4 (2n = 40–52 chromosomes, with many metacentric and submetacentric chromosomes) according to a classification based on molecular data and variation in the diploid number (Oliveira et al., 1992). Considering our results, *Corydoras* sp. (2n = 46, 22 m + 22sm + 2st) is the third karyotyped species of this group occurring in the Iguassu River basin: *C. carlae* (2n = 46, 22 m + 22sm + 2st) collected in the Lower Iguassu River (Rocha et al., 2016), *C. paleatus* (2n = 44, 20 m + 24sm) collected in the Upper Iguassu River (Oliveira et al., 1993), and *C.* aff. *paleatus* (2n = 44, 18 m + 26sm) collected in the Upper Iguassu River (Barbosa et al., 2017) were analyzed early. Thus, the similar karyotypic macrostructure

Table 2 Results of principal component analysis (PCA). For each axis, the eigenvalues, the percent of variance explained, and the broken-stick eigenvalues are given. For each index variable, the eigenvector (loading or correlation) is listed. The number of specimens analyzed was 17 for *Corydoras carlae* and 24 for *Corydoras* sp. *Significant differences were observed for these indexes between the species analyzed (Permanova; $p < 0.05$)

Index	Axis 1	Axis 2
Eigenvalues	4.57	2.76
Broken-stick eigenvalues	3.64	2.64
% of variance	21.79	13.14
Thorax length/standard length	0.24	–0.16
*Abdomen length/standard length	0.15	–0.09
Body height at the origin of the dorsal-fin/standard length	0.37	0.04
Predorsal distance/standard length	0.21	0.38
Prepelvic distance/standard length	0.25	0.27
Preanal distance/standard length	0.05	0.18
Preadipose distance/standard length	0.01	–0.03
Dorsal-fin spine length/standard length	0.02	0.32
Pectoral-fin spine length/standard length	0.27	0.17
Caudal peduncle height/standard length	0.07	0.29
Adipose-fin spine length/standard length	0.08	0.37
Distance between the end of the base of the dorsal fin and the origin of the spine of the adipose fin/standard length	0.01	–0.03
Dorsal-fin base length/standard length	0.07	0.24
Anal-fin base length/standard length	0.04	0.05
Maximum width of the cleiter/standard length	0.05	0.29
Maxillary barbell length/head length	–0.23	–0.03
Head height/head length	–0.22	0.14
*Interorbital distance/head length	–0.39	0.22
*Horizontal orbit diameter/head length	–0.36	0.04
Snout length/head length	–0.28	0.20
Internareal distance/head length	–0.30	0.26

between *C. carlae* and *Corydoras* sp. probably indicates that these species are undergoing a recent speciation process.

The studied specimens of *C. carlae* and *Corydoras* sp. had only one Ag-NOR situated on the long arm of the sixth-largest metacentric pair. Ag-NORs were also evident in a single metacentric pair in a terminal position of the long arm in *C. ehrhardti* and *C. paleatus* (Artoni et al., 2006). According to Oliveira and Gosztonyi (2000), the presence of simple Ag-NORs in the terminal location is a possible basal condition for Siluriformes. Thus, *C. carlae* and *Corydoras* sp., presenting simple Ag-NORs in the terminal location, seem to maintain this basal condition.

FISH analyses revealed that the two *Corydoras* species studied shared the same location and number of 18S sites, with terminal markings in two metacentric chromosomes and syntenic marks with 5S ribosomal sites. The occurrence of one chromosome pair bearing 18S rDNA in *C. carlae* and *Corydoras* sp. is similar to what has been found in *C. ehrhardti* (Artoni et al., 2006; Barbosa et al., 2017) and *C.* aff. *paleatus* (Barbosa et al., 2017). However, only the 18S ribosomal sites of *C. ehrhardti* were syntenic with 5S, albeit in independent clusters, while in *C. carlae* and *Corydoras* sp., the 5S rDNA was interspersed along with the clusters of 18S rDNA (colocalization). Synteny is a rare trait in fish and is recorded here for the first time in *Corydoras* sp., highlighting the originality of these results. Syntenic 18S and 5S markings have already been described for Callichthyidae, *Callichthys callichthys* (Konerat et al., 2014), *C. carlae* (Rocha et al., 2016), and *C. ehrhardti* (Barbosa et al., 2017). Thus, our results expand the synteny information for the 18S and 5S ribosomal sites of the family. The colocalization of 18S and 5S has also been described for other groups of fish, such as *Mugil incilis* (Hett et al., 2011), *Psalidodon fasciatus*, *P. scabripinnis* (Almeida-Toledo et al., 2002), and *Salea senegalensis* (Cross et al., 2006).

In contrast, 5S rDNA is a chromosomal marker that is specific to the species analyzed in this study. The localization of the 5S rDNA sites is divergent among *C. carlae* and *Corydoras* sp. In *Corydoras* sp., the in situ analysis of the 5S rDNA sequences revealed signals on three chromosomes, while only one chromosome pair bearing 5S rDNA was present in *C. carlae*. The syntenic marking of the 18S and 5S rDNA in pair 6 was shared between the two species studied. However, in *Corydoras* sp. additional 5S rDNA cistrons located in pericentromeric position on the short arm of one homolog of pair 15 were detected. From an evolutionary point of view, these data are intriguing because the insertion of transposable elements in sequences of the 5S rDNA of the metacentric pair could have led to the dispersion of these sequences to the submetacentric chromosome (par 15) of *Corydoras* sp. According to Raskina et al. (2004), one of the mechanisms responsible for the process of moving rDNA sequences to new sites would be due to the action of transposable elements. The action of transposable elements was suggested to justify the difference in the number and location of 5S rDNA cistrons in three species of *Bryconamericus* (Piscor et al., 2013) and appears to be responsible for the dispersion of 5S rDNA in almost all chromosomes of *Hyphessobrycon eques* (Piscor et al., 2020).

Corydoras species share a heterochromatin distribution pattern that is very similar, preferably centromeric and pericentomeric, and in most cases is associated with NORs. In *C. carlae* and *Corydoras* sp., this pattern was also observed, with centromeric and pericentromeric heterochromatic blocks displayed on many chromosomes. Thus, the heterochromatin distribution pattern was not an effective marker in the delimitation of the *Corydoras* species analyzed here. *Corydoras britskii* from the Miranda River also showed a large amount of pericentromeric heterochromatin, but with

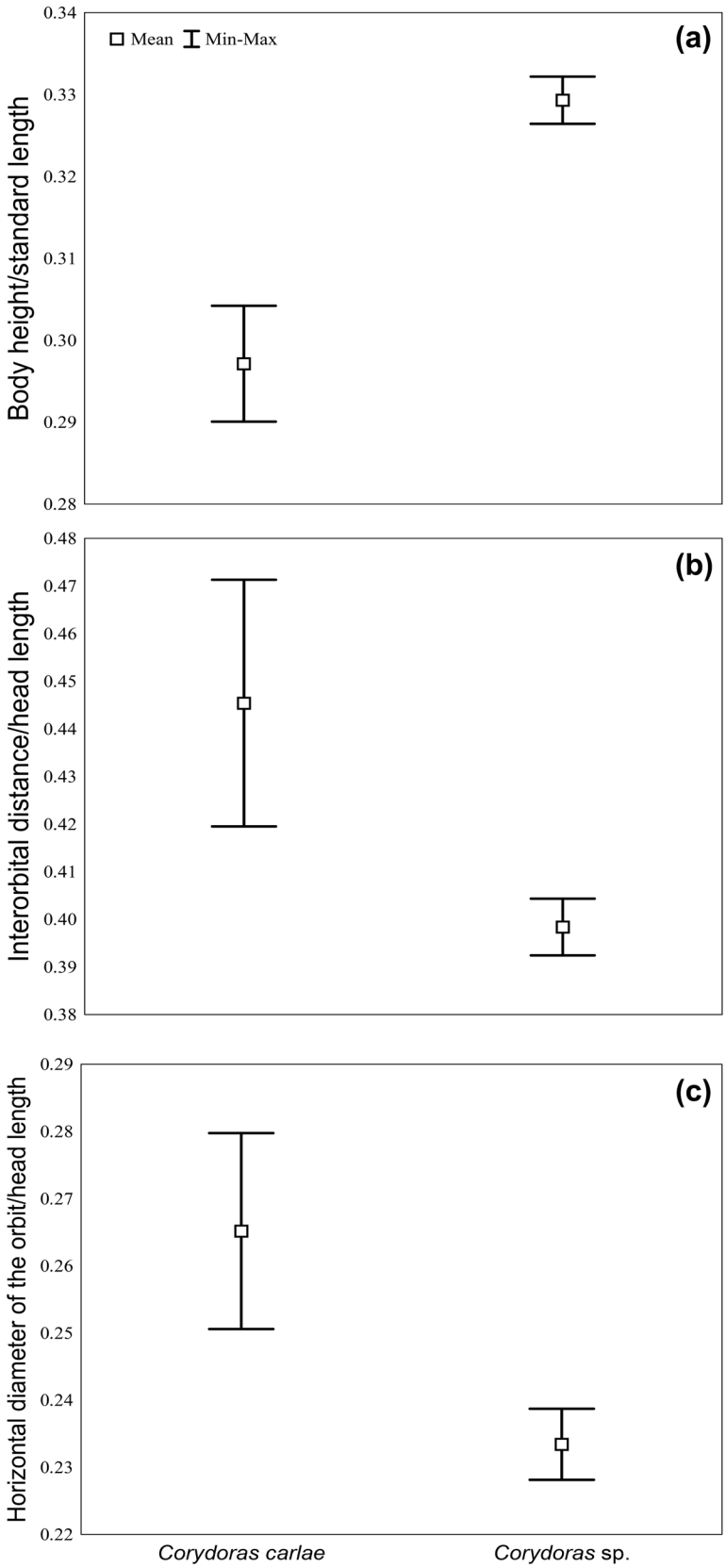


Fig. 5 Principal component analysis scores (mean and maximum and minimum values) for the morphometric data of *Corydoras carlae* samples in the Florido River, and *Corydoras* sp. sampled in the Poço Preto Stream, lower Iguassu River basin. Proportions that showed a significant difference between species: (**a**) body height/standard length; (**b**) interorbital distance/head length; (**c**) horizontal diameter of the orbit/head length

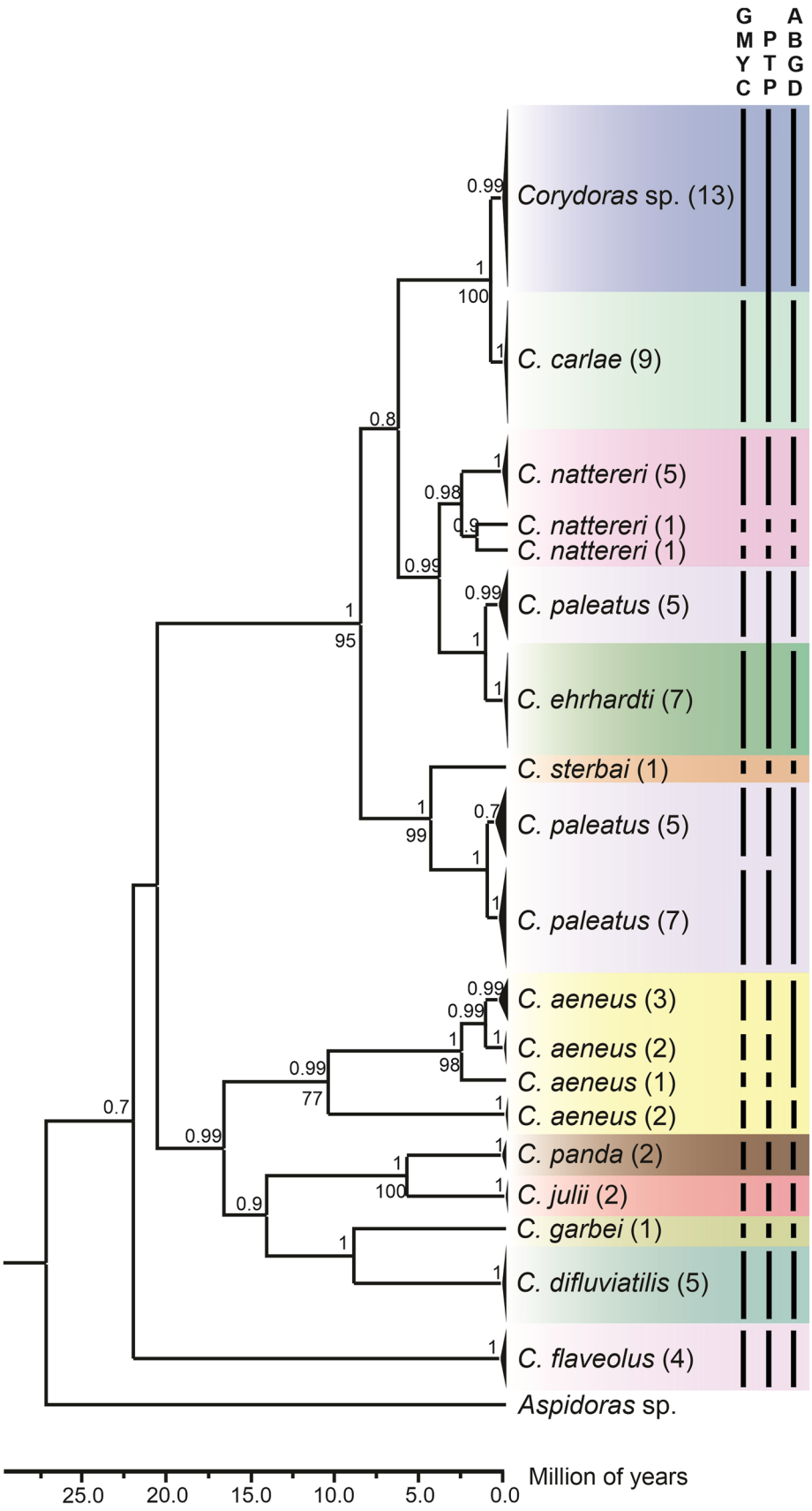
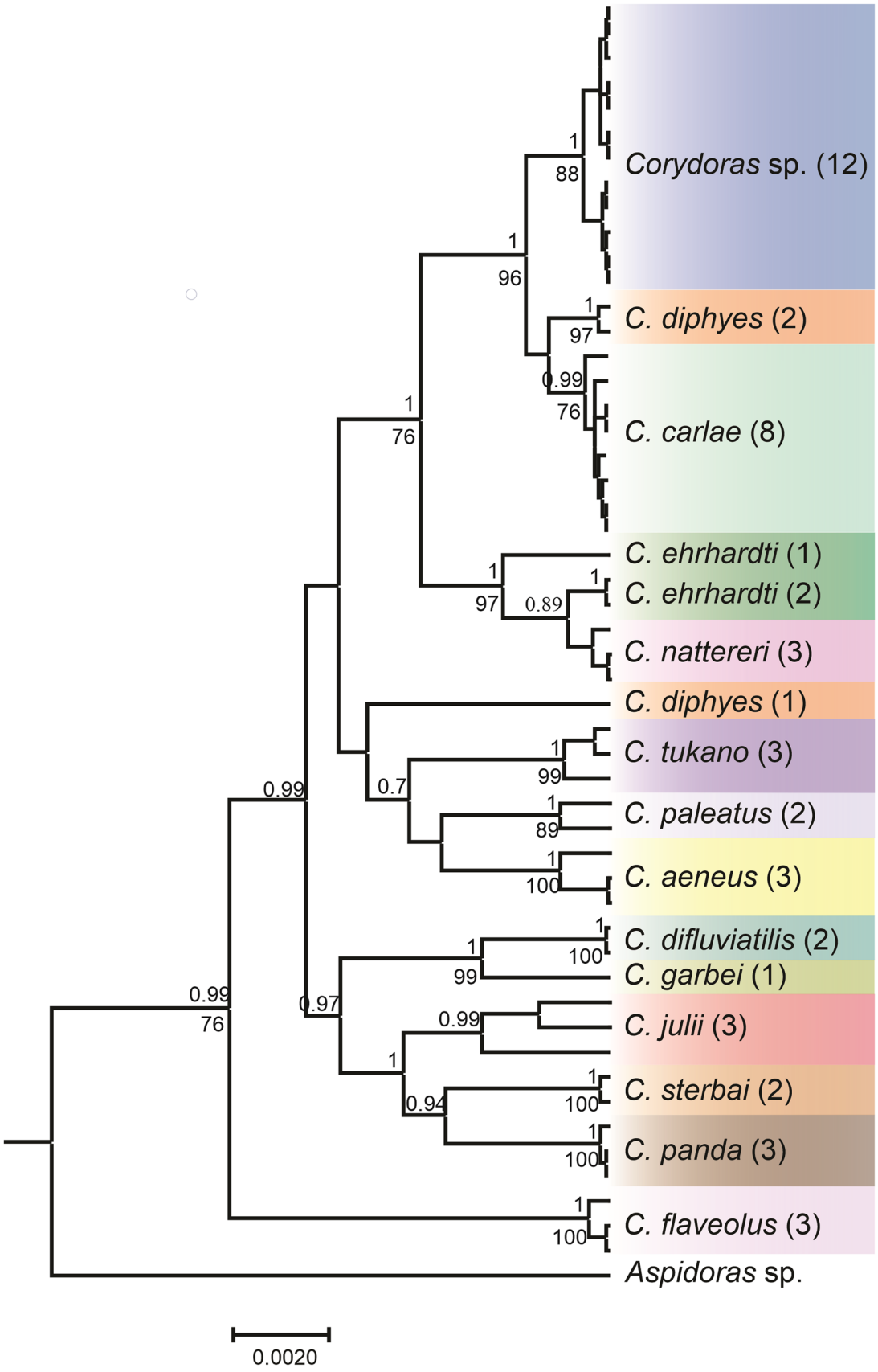


Fig. 6 Calibrated Bayesian tree based on the cytochrome c oxidase I gene sequence of *Corydoras* species. Values in parentheses indicate sample number for each species. Values near branches indicate Bayesian (posterior probability; above) and maximum likelihood (bootstrap; below) support values for each node. The dashed lines indicate the delimitations of molecular operational taxonomic units (MOTUs) according to the GMYC, PTP, and ABGD approaches


Corydoras sp. (12)
C. diphyes (2)
C. carlae (8)
C. ehrhardti (1)
C. ehrhardti (2)
C. nattereri (3)
C. diphyes (1)
C. tukano (3)
C. paleatus (2)
C. aeneus (3)
C. difluviatilis (2)
C. garbei (1)
C. julii (3)
C. sterbai (2)
C. panda (3)
C. flaveolus (3)
Aspidoras sp.
0.0020

◂**Fig. 7** Bayesian phylogenetic tree based on the recombination activating protein 1 (RAG1) gene of *Corydoras* species. Values in parentheses indicate the sample number for each species. Values near branches indicate Bayesian (posterior probability; above) and maximum likelihood (bootstrap; below) support values for each node

terminal heterochromatic blocks (Takagui et al., 2014), which were not observed in this study.

Morphometry

Our results revealed differences between species in morphometric proportions, especially for body height/standard length, interorbital distance/head length, and the horizontal diameter of the orbit/head length. On the other hand, Tencatt (2013) morphologically compared *Corydoras* sp. with *C. carlae* collected in the Tormenta, Adelaide, and Guarani rivers and found that these species can be differentiated by differences in the lengths of their dorsal and pectoral clusters (dorsal cluster 13.7–22.5% in the SL; pectoral cluster 15.1–22.4% in the SL in *Corydoras* sp. vs. 26.6–33.6 and 25.9–31.90 in *C. carlae*).

The differences found make it possible to speculate that these are different species, possibly because both have different geographic distributions. *Corydoras carlae* were collected in the Florido River upstream of Iguassu Falls, while *Corydoras* sp. were captured only in the Poço Preto Stream, downstream of Iguassu Falls. In this way, the two species analyzed are separated by a natural geographic barrier (Iguassu Falls) formed approximately 22 million years ago, which is considered to be one of the main causes of isolation and allopatric speciation of fish species for the Iguassu River basin (Agostinho & Gomes, 1997; Baumgartner et al., 2012; Garavello et al., 1997; Mezzaroba et al., 2021). However, populations apparently corresponding to *C. carlae* were recorded in the Río Urugua-í basin, a tributary of the lower Paraná River in Misiones, Argentina, and thus, considering that they are in fact fragmented populations (probably relictual) of the same species, *C. carlae* would no longer be restricted to tributaries of the Iguassu River upstream of Iguassu Falls.

In a study carried out with *C. paleatus* from different basins, including the Iguassu River (Shibatta & Hoffmann, 2005), it was proposed that the differences found between the populations occurred due to the uplift of Iguassu Falls, which separated the species from the Paraná River and the Iguassu River. On the other hand, Florentino and Súarez (2014) attributed and correlated the differences between populations of *C. aeneus* to the characteristics of the environment that, over evolutionary time, selected the individuals with the greatest adaptation. According to the evidence presented by Tencatt et al. (2016), these populations, although often separated by well-defined geographical barriers, apparently correspond to a single and variable species, *C. longipinuunis*.

Molecular analysis

Molecular analyses revealed a separation between *C. carlae* and *Corydoras* sp. (Figs. 5 and 6), presented exclusive haplotypes (or groups of haplotypes), allowed correct discrimination of species and provided evidence for the nonsharing of haplotypes, which also suggested the absence of gene flow. In addition, two of the three species delimitation methods (GMYC and ABGD) assigned two different MOTUs to *Corydoras* species from the Iguassu River. Although these data show a clear separation of the two species, the average value of genetic distance was not sufficient for their discrimination, according to the threshold stipulated by the DNA barcoding methodology.

DNA barcoding is one of the most commonly used tools today in the identification of species based on DNA sequences (Hebert et al., 2003). The methodology is based on a standardized region of the mitochondrial cytochrome c oxidase I (COI) gene for the identification of animal species based on differences in their COI sequences (Hebert et al., 2003). A threshold value of 2% divergence is normally used in the delimitation of species (Carvalho et al., 2011; Pereira et al., 2011; Ward, 2009; Ward et al., 2009). Although the method is highly efficient in identifying a large number of animal species, including fish (e.g., Carvalho et al., 2011; Hubert et al., 2008; Pereira et al., 2011, 2013), criticisms have been made regarding the use of a single gene to delimit species and, mainly, regarding the established cutoff value. Most likely, the 2% COI divergence threshold is not suitable for all groups of fish, especially for some pairs of species that may naturally have low interspecific values, as is the case with fish from the Neotropical region (Pereira et al., 2013).

Although *C. carlae* and *Corydoras* sp. have an average value of interspecific distance K2P (1.13%; Table 3) below the barcode threshold of 2%, the species formed cohesive groups of haplotypes and presented diagnostic nucleotides (i.e., species-specific; Table S2) (Wong et al., 2009), which allowed the correct identification of species based not only on COI sequences but also on RAG1. In addition, even though the average interspecific K2P genetic distance values were low (1.13% for COI; 0.49% for RAG1), the intraspecific average values were comparatively many times smaller (0–0.02% for COI; 0–0.01% for RAG1). Furthermore, the nonmonophyletic conditions observed for *C. carlae* and *Corydoras* sp. (data based on RAG1 sequences; Fig. 6) reinforce the hypothesis that they are different species.

Similarly, Pereira et al. (2013) found several pairs of neotropical fish species with low genetic distance values for the COI gene (< 2%). However, correct delimitation of the species was possible due to the formation of cohesive groups of haplotypes, as well as the occurrence of diagnostic

Table 3 Average values of genetic distance (K2P), shown as percentages, between *Corydoras* species based on the partial nucleotide sequences of the COI (above the diagonal) and RAG1 (below the diagonal) genes. Diagonally, average distance values within the species of *Corydoras*

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *Corydoras* sp.	0.02	1.13	7.17	13.70	11.74	13.62	14.28	5.65	5.71	12.18	11.99	12.98	–
2. *C. carlae*	0.49	0.00	7.85	13.81	12.15	14.25	13.86	6.20	6.84	12.99	11.99	12.57	–
3. *C. paleatus*	1.76	1.92	2.93	13.61	7.03	14.03	14.19	4.40	5.52	13.14	11.94	13.46	–
4. *C. aeneus*	2.50	2.60	1.89	4.91	17.40	12.71	13.32	13.20	13.68	14.20	11.79	12.25	–
5. *C. sterbai*	3.21	3.36	3.33	3.99	–	18.12	17.27	9.82	10.16	18.38	16.22	17.11	–
6. *C. panda*	2.31	2.47	2.29	3.07	2.50	0.33	6.59	13.27	14.64	13.88	11.45	11.33	–
7. *C. julii*	2.32	2.47	2.18	2.55	2.87	1.88	0.00	13.31	14.38	14.34	11.29	10.97	–
8. *C. ehrhardti*	1.81	1.96	2.42	3.26	3.93	2.93	3.26	0.00	3.84	11.90	11.44	13.32	–
9. *C. nattereri*	1.44	1.60	2.10	2.89	3.55	2.65	2.94	0.74	1.77	13.15	13.32	14.12	–
10. *C. flaveolus*	2.45	2.61	2.56	2.36	3.60	3.39	3.03	3.16	2.79	0.21	14.52	14.08	–
11. *C. garbei*	2.86	2.88	3.11	3.91	3.88	3.53	3.49	3.85	3.48	3.62	–	8.42	–
12. *C. difluviatilis*	3.00	3.03	2.98	3.78	4.32	3.40	3.51	3.62	3.34	3.67	1.88	–	–
13. *C. diphyes*	1.36	1.38	2.20	2.80	3.56	2.93	2.81	2.46	2.16	3.08	3.39	3.49	–
14. *C. tukano*	1.71	1.87	1.57	2.48	3.14	2.38	2.44	2.38	2.06	2.73	3.11	3.35	2.34

nucleotides. Maia (2014), in a study aimed at molecular identification of fish specifically from Corydoradinae, obtained the correct discrimination of 85% of 94 species analyzed using DNA barcoding methodology. However, seven pairs of species had a genetic distance value of less than 2% (Maia, 2014), reinforcing the hypothesis that some groups of fish have low genetic distance values. In this study, all pairs of *Corydoras* species analyzed had genetic distance values above the 2% threshold for the barcode sequences (Table 3). The only exception was the distance between *C. carlae* and *Corydoras* sp.

A possible explanation for the low values of genetic distance found may be related to recent radiation. Based on previous studies, most of the diversification of Neotropical ichthyofauna occurred recently, 10-3 Mya (Hubert et al., 2007; Lovejoy & Araújo, 2000; Montoya-Burgos, 2003; Pereira et al., 2013). According to the estimated divergence time obtained, *C. carlae* and *Corydoras* sp. started to differentiate approximately 1.1 Mya. The recently estimated origin for *C. carlae* and *Corydoras* sp. is consistent with the low genetic distance values identified between species. These species are probably undergoing a recent speciation process, which has prevented further accumulation of polymorphisms in the DNA. According to Queiroz (2007), the speciation process is not uniform, and depending on the character evaluated, it is possible not to reach a precise conclusion regarding the existence of one or more species. This moment of uncertainty during speciation is called the gray zone (Queiroz, 2007), and *C. carlae* and *Corydoras* sp. may be going through this period. According to the results obtained so far, *C. carlae* and *Corydoras* sp. appear to correspond to independent evolutionary lineages.

The fact that *C. carlae* and *Corydoras* sp. do not occur together must have contributed to establishing the morphological, cytogenetic, and genetic differences found between them, since the lack of gene flow between species supposedly resulted in a process of speciation. The differences found between the two species of *Corydoras* analyzed are probably due to the geographic isolation caused by the uplift of Iguassu Falls. In addition to this notable geographical barrier, others occur along the Iguassu River, such as Salto Saicanga, Salto Grande, Salto Santiago, Salto Osório, and Salto Caxias (Maack, 2012), almost all flooded by the formation of reservoirs (Baumgartner et al., 2012). However, the differentiation of the species must have occurred after the uplift of Iguassu Falls, since low values of interspecific genetic distance and recent divergence times were detected (1.1 Mya). Geographic isolation is also suggested to explain cytogenetic differences, which is reflected in the diploid number and banding patterns of fish species such as *Characidium* in the Paraná River basin (Pucci et al., 2014), *Psalidodon scabripinnis* (=*Astyanax scabripinnis*) (Moreira-Filho & Bertollo, 1991), and *Astyanax lacustris* (=*Astyanax altiparanae*) (Hashimoto et al., 2008).

In this context, the evolution of fish species confined to different hydrographic systems is the result of a close relationship between the histories of the basins and the evolutionary histories of their species (Kavalco & Moreira-Filho, 2003). An interesting feature is that small fish species tend to be more susceptible to speciation, since populations located in small streams can diverge genetically from the others more quickly than the typical species of large rivers (Weitzman et al., 1998). However, from a genetic perspective, speciation caused by reproductive isolation is a property of a few

individual loci or genomic regions and not of the genome as a whole (Lexer & Widmer, 2008; Qvarnstron & Bailey, 2009).

Although *C. carlae* and *Corydoras* sp. have the same diploid number, karyotype formula, number of Ag-NORs, and the same pattern distribution of constitutive heterochromatin, differences in the number of chromosomes carrying 5S rDNA cistrons were observed between species. In addition, the synteny and colocalization of 5S rDNA with 18S rDNA represent unprecedented results for *Corydoras*. Nevertheless, analysis of the morphometric proportions also confirmed significant differences between the species. The combined analyses of cytogenetic, morphometric, and molecular results were important for characterization of the two species and made it possible to differentiate *C. carlae* from *Corydoras* sp. via an allopatric speciation process, indicating they are distinct cytogenetically, molecularly, and morphologically. Therefore, there is evidence that *C. carlae* and *Corydoras* sp. of the Iguassu River basin comprise distinct evolutionary lineages that are probably undergoing a recent process of speciation.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s13127-021-00534-8.

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Author contribution ÉAG, LFCT, WJG and RHR conceived the study for this manuscript with input from CAF and VPM. RHR and ÉAG collected data and resources. Most raw data analysis was performed by RHR, TSB, VPM, LFCT and WJG. RHR wrote the first draft, and all authors contributed to writing.

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Availability of data and material Data generated or analyzed during this study consist of 24 fish specimens. Voucher specimens were deposited in the fish collection of the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (NUPELIA), Universidade Estadual de Maringá, Paraná, Brazil, as *Corydoras carlae* (NUP 17885) and *Corydoras* sp. (NUP 14261 and NUP 17887). In addition, all nucleotide sequences generated in this study were deposited in GenBank (GenBank accession numbers = MT846090—MT846111 for COI sequences; MT855475—MT855494 for RAG1 sequences); all GenBank accession numbers used in our analysis are also listed individually in the Table S1.

Code availability Not applicable.

Declarations

Ethics approval This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, approved by the Committee on the Ethics of Animal Experiments of the Universidade Estadual do Oeste do Paraná (License Number: Protocol 13/09 – CEUA/Unioeste).

Consent for publication All authors approved the final version of the manuscript for publication.

Conflict of interest The authors declare no competing interests.

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