Received: 11 September 2019 | Revised: 1 April 2020 | Accepted: 5 April 2020

DOI: 10.1111/jzs.12389

ORIGINAL ARTICLE

JOURNAL OF ZOOLOGICAL SYSTEMATICS AND EVOLUTIONARY RESEARCH

WILEY

Phylogenetic structure of Neotropical annual fish of the genus *Cynopoecilus* (Cyprinodontiformes: Rivulidae), with an assessment of taxonomic implications

Daiana Kaster Garcez[1] | Murilo de Oliveira Fernandes[1] | Gabrielli da Rosa Ozório[1] | Matheus Vieira Volcan[2] | Lizandra Jaqueline Robe[1,3]

[1]Programa de Pós-Graduação em Biologia de Ambientes Aquáticos Continentais, Universidade Federal do Rio Grande, Rio Grande, Brazil

[2]Laboratório de Ictiologia, Instituto Pró-Pampa, Pelotas, Brazil

[3]Departamento de Ecologia e Evolução (DEE), Centro de Ciências Naturais e Exatas (CCNE), Universidade Federal de Santa Maria, Santa Maria, Brazil

Correspondence
Lizandra Jaqueline Robe, Programa de Pós-Graduação em Biologia de Ambientes Aquáticos Continentais, Universidade Federal do Rio Grande, Rio Grande, Rio Grande do Sul, Brazil.
Email: lizbiogen@gmail.com

Funding information
Fundação Grupo Boticário de Proteção à Natureza, Grant/Award Number: 1090_20171; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Grant/Award Number: Finance Code 001

Abstract

The definition of species boundaries constitutes an important challenge in biodiversity studies. *Cynopoecilus* Regan, 1912 encompasses several endangered species of annual fish, occurring in temporary ponds in a restricted area of Southern Brazil and Uruguay. Divergences about the taxonomic status of *Cynopoecilus* species highlight the importance of species delimitation studies. Therefore, we address here the phylogenetic structure of *Cynopoecilus*, while assessing its taxonomic implications. For this, fragments of the mitochondrial *COI* and nuclear *RAG1* genes were characterized and analyzed for a set of 275 and 280 specimens, respectively. DNA barcoding and phylogenetic analyses detected subdivision of these specimens in 8–10 clusters, which comprise the six previously described species, and suggest one invalid taxon and at least 3–5 putative new species. The phylogenetic structure also suggests that the Jacuí River and the Patos Lagoon historically acted as effective barriers to gene flow between populations, although some isolated dispersal events across these water bodies could be evidenced, especially for *C. melanotaenia* Regan, 1912. In general, the results highlight the need of independent conservation strategies within the distribution area of each of the endemic allopatric killifish clusters, while questioning several taxonomic boundaries and distribution data.

KEYWORDS

allopatric fragmentation, cryptic speciation, DNA barcoding, killifish, phylogeography

1 | INTRODUCTION

One of the first steps in any biological study encompasses the evaluation of existing diversity, in order to identify previously described species, while detecting and diagnosing new species. The DNA barcoding technique proposes the use of a fragment of the *cytochrome c oxidase I* mitochondrial gene (*COI*) as a shortcut to assist in these goals (Hebert, Ratnasingham, & Waard, 2003). The efficiency of DNA barcoding approaches depends on the assumption that the intraspecific genetic diversity found for this gene is lower than the interspecific genetic divergence (Meyer & Paulay, 2005), which is largely dependent on the presence of reciprocal monophyly among species (Kekkonen & Hebert, 2014; Meyer & Paulay, 2005). Although both assumptions do not always apply (Funk & Omland, 2003; Meyer & Paulay, 2005), this technique has been playing an important role in the discovery of new species in different animal groups (Hebert, Penton, Burns, Janzen, & Hallwachs, 2004; Kiontke et al., 2011;

Contributing authors: Daiana Kaster Garcez (daiana_kg@hotmail.com), Murilo de Oliveira Fernandes (murilo_fernan@hotmail.com), Gabrielli da Rosa Ozório (gabiiozorio@gmail.com), Matheus Vieira Volcan (matheusvolcan@hotmail.com)

Machado et al., 2017; Witt, Threloff, & Hebert, 2006), including annual fish (Costa & Amorim, 2011; Costa, Amorim, & Aranha, 2014; Costa, Amorim, & Mattos, 2016). In this way, it presents important applications on the biology of conservation, contributing in the assignment of taxonomic status and distribution areas (Pfeiler, 2018), while also assisting in the discovery of cryptic diversity (Kekkonen & Hebert, 2014). Nevertheless, as the reliance on a single marker may impose multiple limitations to the technique (Moritz & Cícero, 2004; Packer, Gibbs, Sheffield, & Hanner, 2009; Song, Buhay, Whiting, & Crandall, 2008), the simultaneous use of a nuclear marker may be advisable, especially if species subdivision is to be assessed. In fact, analysis of a single locus may not be robust enough to propose reliable species hypotheses (Puillandre, Lambert, Brouillet, & Achaz, 2012).

Annual fishes of the family Rivulidae comprise one of the most diverse clades of Neotropical freshwater fishes and are particularly affected by the loss and fragmentation of the ephemeral wetlands they inhabit (Costa, 2002a, 2008; Volcan, Lanés, Gonçalves, & Guadagnin, 2015). This family encompasses about 350 described species, a third of which occur in Brazilian territories (Costa, 2008; Loureiro et al., 2018). At least part of this diversity is explained by the occurrence of geomorphological events that created opportunities for allopatric diversification, through the frequent isolation of small populations (Reichard, 2015). Among the geomorphological events that occurred in Uruguay and southern Brazil, the paleoclimatic alternations of Quaternary caused sea-level variations, opening and closing areas of communication with the Atlantic Ocean. This built a system called as Multiple Barrier (Villwock & Tomazelli, 2007), formed by four major depositional events (Barrier I–IV) that extended from 400,000 to 5,000 years ago and led to the formation of the Patos, Mirim, and Mangueira lagoons.

Allopatric speciation in annual fish may have been further enhanced by large water bodies and even terrestrial areas acting as effective barriers to gene flow (Bartáková, Reichard, Blažek, Polačik, & Bryja, 2015; Bartáková et al., 2013) and interrupting gene flow even between recently diverged and geographically close populations (Barbosa, Garcez, Volcan, & Robe, 2020). In this way, it is not rare to find high levels of cryptic diversity within different species of annual fish, which may actually comprise species complexes (Garcez et al., 2018; García, Gutiérrez, Ríos, & de Sá, 2015; García et al., 2019). Unfortunately, the fact that annual fish usually encompass small and isolated populations also turns these species one of the most endangered taxa. This is further complicated by the straightforward degradation of their habitats (Volcan & Lanés, 2018; Volcan et al., 2015), whether through the landfill of ponds for agriculture or urbanization, or through the release of pollutants that decimate entire populations. In fact, annual fish encompass about 30% of the threatened ichthyofauna in Brazil (ICMBio, 2018).

Among Neotropical rivulids, *Cynopoecilus* Regan, 1912 (described in Regan (1912b)) stands out for having internal fertilization and exhibiting elaborated inseminating structures (Costa et al., 2016). Furthermore, *Cynopoecilus* may be easily diagnosed by the unique color pattern occurring in all species, consisting of a dark reddish-brown to black stripe along the lateral midline of the body and another between the pectoral-fin base and the posterior end of the anal-fin base (Costa, 2002; Costa et al., 2016; Ferrer, Wingert, & Malabarba, 2014). Currently, this genus comprises six endemic species distributed in the coastal plains of the Patos-Mirim Lagoon System and Jacuí River basin, in southern Brazil and Uruguay (Costa et al., 2016; Ferrer et al., 2014): *C. melanotaenia* Regan, 1912 (described in Regan (1912a)), *C. intimus* Costa, 2002 (described in Costa (2002b)), *C. nigrovittatus* Costa, 2002 (described in Costa (2002b)), *C. notabilis* Ferrer et al., 2014, *C. feltrini* Costa et al., 2016, and *C. fulgens* Costa, 2002 (described in Costa (2002b)). This number of species applies after the taxonomic revision performed by Costa (2016), which suggested *C. multipapillatus* Costa, 2002 (described in Costa (2002b)) as a synonym of *C. fulgens*. Among the described species, *C. intimus*, *C. fulgens*, and *C. nigrovittatus* have the status of vulnerable to extinction (SEMA, 2014; Volcan et al., 2015). Although *C. notabilis* is known only from its type locality, it is not included in the red list because it occurs within a conservation unit, which is considered to reduce the immediate likelihood of anthropogenic impacts (Ferrer et al., 2014).

In this study, our aim was to address the phylogenetic structure across the genus *Cynopoecilus*, while addressing its taxonomic implications. In this sense, we applied the phylogenetic species concept to address the hypothesis that some species of the genus are subdivided into cryptic or still unrecognized lineages, deserving further descriptions, while others are merged in a unique evolutionary lineage, deserving synonymizations. As a consequence of allopatric fragmentation, we believe that species of *Cynopoecilus* inhabiting areas in which recent geomorphological events have occurred (as *C. fulgens*), or possessing geographic barriers in its current distribution area (as *C. nigrovittatus*), may be especially prone to present cryptic diversity. The refinement of these questions is, thus, fundamental to assess the conservation status of every *Cynopoecilus* species and to delimit hot spots to the conservation of the genus.

2 | MATERIAL AND METHODS

2.1 | Sampling

This study includes molecular data from a total of 280 individuals from the six species of *Cynopoecilus*, 242 of which were collected between 2014 and 2018 across 34 sampling locations (Table S1), distributed in the entire known distribution range of the genus (Costa, 2002b; Costa et al., 2016; Ferrer et al., 2014) (Figure 1). Additionally, 11 sequences were obtained from GenBank (accession numbers KT823671.1–KT823674.1, KT823652.1–KT823655.1, KT823656.1–KT823658.1, and KT590068.1–Table S1) and 27 tissues were made available by Departamento de Zoologia, Universidade Federal do Rio Grande do Sul, Porto Alegre (UFRGS). Sequences of *Austrolebias wolterstorffi* Ahl, 1924 (accession numbers KY093034.1 and KJ844671.1), *Nematolebias whitei* Myers, 1942 (accession number KJ844686.1), and *Simpsonichthys igneus* Costa, 2000 (accession

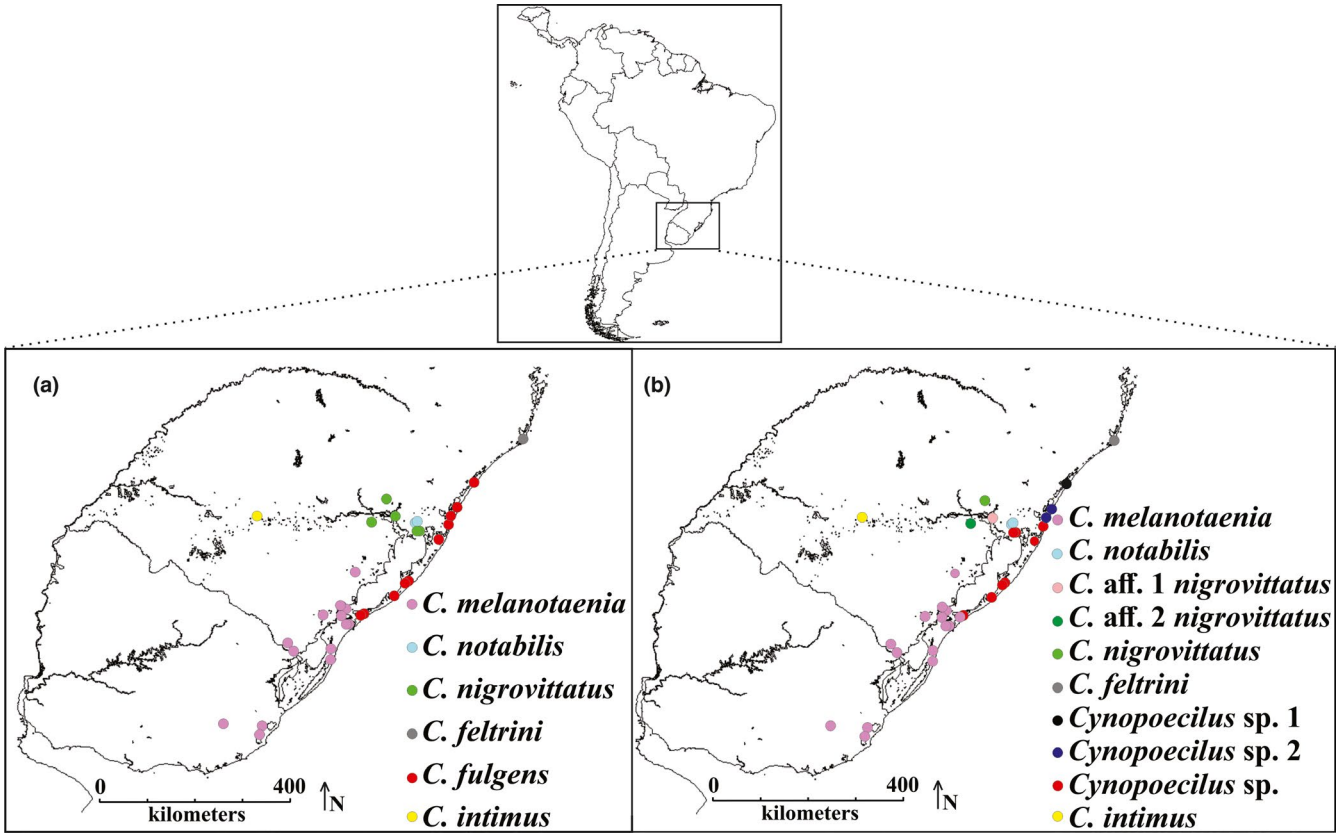

FIGURE 1 Distribution map of the 34 sampling sites of *Cynopoecilus*, emphasizing differences between traditional species boundaries (a) and lineages defined after this study (b)

number HQ833482.1) were included in the analyses as out-group taxa. Voucher material was deposited in the Fish Collection of the Museu de Ciências e Tecnologia of the Pontifícia Universidade Católica do Rio Grande do Sul (MCT-PUCRS) (MCP 54235-MCP 54,254–Table S1).

The fishes were collected in temporary ponds with the help of hand nets, euthanized with an overdose of 3,000 mg/L of eugenol anesthetic and then fixed in 95% ethanol. All collections were authorized by the Sistema de Autorização e Informação em Biodiversidade (SISBIO) of the Brazilian Ministério do Meio Ambiente (MMA) and by the Ethics Committee on Animal Use of the Universidade Federal do Rio Grande (CEUA-FURG).

2.2 | DNA manipulation

Total DNA was extracted from each specimen from approximately 30 mg of muscular tissue, using a phenol/chloroform protocol (Sambrook, Fritschi, & Maniatis, 1989), or when necessary, using the DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer instructions. A total of 1,182 bp of the mitochondrial *cytochrome c oxidase subunit I* (*COI*) gene and 562 bp of the *recombination activating protein 1* (*RAG1*) gene were amplified from each sample using the primers LCO1490 (GGTCAACAAATCATAAAGATATTGG) and COIb (CCAGAGAATAGAGGGAATCAGTG) (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994 and Murphy, Thomerson, & Collier, 1999, respectively) and L2891_RAG1ex3 (AAGGAGTGYTGYGATGGCATGGG) and H3405_RAG1ex3 (GCNGAGACTCCTTTGACTCTGTC) (Near et al., 2012), respectively. PCRs were carried out using 100 ng of DNA in 25 µl reactions, containing 1 × buffer, 0.3–0.5 µM of each primer, 0.30 mM of each dNTP, 2.5–3 mM of $MgCl_2$, and 1–1.5 U of Taq DNA polymerase. PCR conditions for *COI* consisted of an initial stage of denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 55 to 51°C for 40 s and extension at 72°C for 70 s, and by a final extension stage at 72°C for 10 min; for *RAG1*, cycling consisted of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. After an electrophoresis performed to confirm the success of amplification, amplicons were purified with a solution of 7.5 M ammonium acetate ($C_2H_7NO_2$) and directly sequenced by MACROGEN (Seoul, Korea).

2.3 | Matrix construction

First, contigs were assembled and inspected using the Gap4 software of the Staden package (Staden, 1996). The sequences were then aligned using the Clustal W algorithm in MEGA 7.0 software (Kumar, Stecher, & Tamura, 2016). For *RAG1*, heterozygous sites were coded according to the nucleotide degeneracy/redundancy table and later unphased in DnaSP 5.10 software (Librado &

Rozas, 2009). This software was also employed to measure the minimum number of recombination events that best explains the diploid dataset.

2.4 | Phylogenetic and coalescence analyses

We used different strategies related to the DNA barcoding approach, employing only *COI* or *RAG1*, or even the concatenated dataset, in order to delimit taxonomic units and evaluate the presence of cryptic diversity. At first, phylogenetic analyses were performed through Bayesian inferences (BI) to evaluate questions of reciprocal monophyly, and to test the validity of the six species of *Cynopoecilus* proposed so far. These analyses were performed in MrBayes 3.2.6 (Ronquist et al., 2012) under the best substitution model suggested by a BIC (Schwarz, 1978) test performed in jModelTest 2.1.10 (Posada, 2008). The Markov chain Monte Carlo (MCMC) of the BI was run for 10,000,000 generations, sampling trees every 1,000 generations, and burning 25% of the initial results. These analyses were performed with the use of out-groups, and the phylogenetic trees were further visualized and edited in FigTree 1.4.3 (Rambaut & Drummond, 2009).

The general mixed Yule coalescent (GMYC) (Pons et al., 2006) analysis was also employed to test species assignments, without a priori species hypotheses. This approach relies on the detection of transitions between intra- and interspecific evolutionary processes, that is, between coalescence and cladogenesis (Pons et al., 2006). This analysis was performed in SPLITs (species limits by threshold statistics; Monaghan et al., 2009), with RStudio (http://r-forge.r-project.org/ projects/splits), after reconstruction of three ultra-metric trees under different molecular clock and tree models in BEAST v.1.7.5 (Drummond, Suchard, Xie, & Rambaut, 2012): (a) Yule model, with a constant speciation rate and a strict molecular clock; (b) Coalescent model, with constant population size and a strict molecular clock; and (c) Coalescent model, with constant population size and a relaxed molecular clock. In all cases, substitution models were selected under a BIC test, performed in the jModelTest 2.1.10, and the concatenated analysis was performed with unlinked substitution models. As we just needed simple ultra-metric trees with relative divergence times, default clock.rates and ucld.mean values were employed. The three independent runs were carried out with 20 million generations each, sampling every 2,000, and burning 10% of initial results. Data mixing and effective sample size (ESS) were checked in Tracer v1.7.1 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018), and TreeAnnotator was used to summarize the posterior distribution.

Finally, the relationships between haplotypes or alleles were inferred from networks generated by median joining in the Network v.4.510 software (Bandelt, Forster, & Röhl, 1999). For *RAG1*, this analysis was preceded by the deletion of sites with recombination signals.

2.5 | Distance analyses

The levels of genetic differentiation among species or lineages were measured by the fixation index (FST) in Arlequin 3.5 (Excoffier & Lischer, 2010) using pairwise differences with 10,000 random permutations. Furthermore, mean pairwise distances between lineages were measured in MEGA 6 using the Kimura 2-Parameters (K2P) (Kimura, 1980) evolutionary model.

We also employed the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) algorithm to test species assignments under distance approaches, without a priori species hypothesis. For this analysis, the aligned sequences of each matrix were uploaded to the Web site http://wwwabi.snv.jussieu.fr/public/abgd/ and then evaluated under K2P distances in order to detect significant differences between intra- and interspecific variations (barcoding gaps) (Zimmermann, Campos-Filho, Deprá, & Araujo, 2015).

2.6 | Migration analysis

Indirect measures of migration rates within the major structure suggested by the phylogenetic analysis were obtained in LAMARC 2.1.10 (Kuhner, 2006) using *COI* sequences. Runs consisted of two simultaneous likelihood searches, each with 10 initial and four final chains, with a minimum of 1,000 and 10,000 recorded parameter sets, respectively, sampling every 20 generations after a burn-in of 1,000 genealogies.

3 | RESULTS

The present study characterized 264 new partial mitochondrial *COI* sequences of 982 bp (120 from *C. melanotaenia*, 14 from *C. intimus*, 68 from *C. nigrovittatus*, three from *C. notabilis*, six from *C. feltrini*, and 53 from *C. fulgens*) and 280 new partial nuclear *RAG1* sequences of 432 bp (119 from *C. melanotaenia*, 14 from *C. intimus*, 72 from *C. nigrovittatus*, eight from *C. notabilis*, 11 from *C. feltrini*, and 56 from *C. fulgens*), from a total of 34 populations (Figure 1). As 11 sequences of *COI* (four from *C. melanotaenia*, one from *C. notabilis*, and six from *C. fulgens*) were downloaded from GenBank, our analyses totaled 275 and 280 specimens of *Cynopoecilus* for *COI* and *RAG1*, respectively (Alignments S1 and S2). All sequences generated in this study were deposited in GenBank (*COI*, MK836466-MK836725 and MN326791-MN326794; *RAG1*, MK836726–MK837005) (Table S1).

3.1 | Phylogenetic, coalescence, and migration analyses

Bayesian phylogenetic analyses produced trees with congruent topologies for both *COI* considered individually (Figure 2a) or in combination with *RAG1* (Figure 2c). In both cases, only *C. intimus*, *C. notabilis*, and *C. feltrini* revealed reciprocally monophyletic. In fact, *C. melanotaenia* revealed paraphyletic with regard to some individuals of *C. fulgens*, whereas *C. fulgens* and *C. nigrovittatus* revealed polyphyletic. Moreover, as individuals from the type locality of *C. fulgens* (São José do Norte, locality 3—RS, Costa, 2002b)

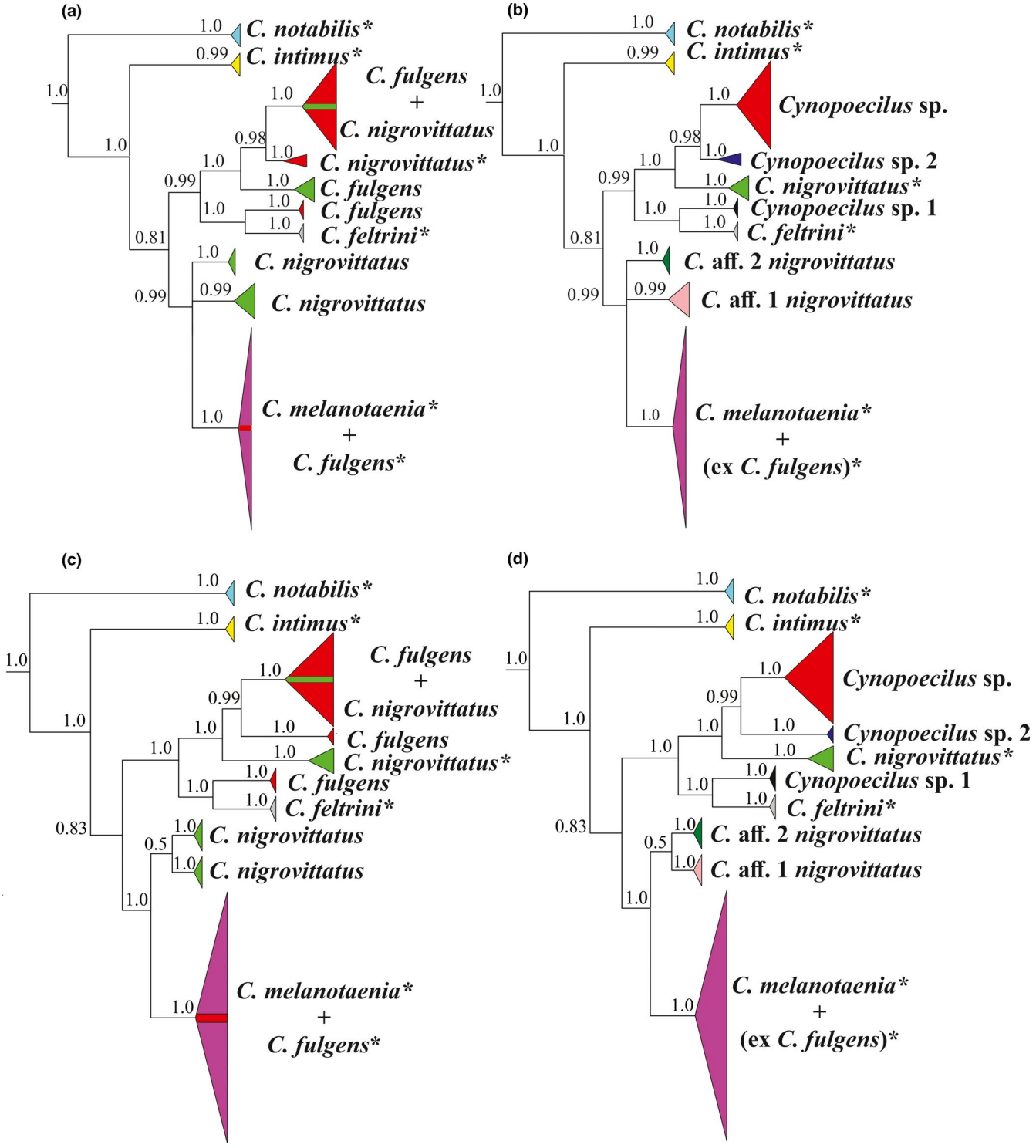

FIGURE 2 Tree topology generated by Bayesian Inference of *COI* (a, b) and *COI* + *RAG1* (c, d) sequences of *Cynopoecilus* emphasizing differences between traditional species boundaries (a, c) and lineages defined after this study (b, d). Numbers above branches refer to the Bayesian posterior probability of each clade. The asterisks indicate location of the type specimens. Colors are in accordance with distribution patterns presented in Figure 1

appeared intermingled among individuals of *C. melanotaenia*, we hereafter question the taxonomic validity of *C. fulgens* and call other populations of this species as *Cynopoecilus* sp. (Figure 2b,d).

Without a priori species assignments and employing only the *COI* matrix or the concatenated dataset, GMYC supported the subdivision of *Cynopoecilus* in 10 clusters, which were considered as independent taxonomic units in further analyses: (1) *C. melanotaenia*, which includes all populations of *C. melanotaenia* plus all individuals previously assigned to *C. fulgens* (i.e., ex *C. fulgens*) collected in São José do Norte locality 3 and some individuals collected in São José

do Norte locality 4; (2) *C. intimus* from Santa Maria; (3) *C.* affinis 1 *nigrovittatus* from the population of Eldorado do Sul; (4) *C.* affinis 2 *nigrovittatus* from the population of Arroio dos Ratos; (5) *C. nigrovittatus* from the population of Montenegro, the type locality of the species; (6) *C. notabilis* from the type locality, Águas Claras; (7) *C. feltrini* from the type locality, Laguna; (8) *Cynopoecilus* sp. 1 ex *C. fulgens* from Torres; (9) *Cynopoecilus* sp. 2 ex *C. fulgens* from Xangri-lá and Osório; and (10) *Cynopoecilus* sp. with seven populations of ex *C. fulgens* [including some individuals of São José do Norte locality 4] and two populations from Viamão previously identified as *C. nigrovittatus*.

All these 10 clusters were recovered as reciprocally monophyletic by the BI trees reconstructed individually with *COI* or simultaneously for *COI* + *RAG1*, with support values higher than 0.99 (Figure 2b,d). The sole difference between these trees was that the polytomy recovered for *C.* aff. 1 *nigrovittatus* and *C.* aff. 2 *nigrovittatus* by *COI* (Figure 2b) resolved as a single clade by the concatenated dataset (Figure 2d), raising the possibility that these two clusters may encompass a single lineage. Moreover, each of the 10 clusters was recovered as independent haplogroups in the *COI* network (Figure 3a–b). These were interconnected by one to 65 step-mutations, without a single case of haplotype sharing. Conversely, *RAG1* network (Figure 3c–d) recovered only two clusters as independent haplogroups (*C. notabilis* and *C.* sp. 1), with straightforward haplotype sharing among the other eight clusters and a general star-like pattern.

Notably, in the phylogenetic trees (Figure 2b,d), cluster 6 (*C. notabilis*) constituted the early offshoot within the genus, followed by the branching of cluster 2 (*C. intimus*). Interestingly, the other eight well-supported clusters branched out to divide into two main clades encompassing populations inhabiting drainages located at the western and/or south (cluster of *C. melanotaenia*, *C.* aff. 1 *nigrovittatus*, and *C.* aff. 2 *nigrovittatus*) and eastern and/or north (*C. nigrovittatus*, *C. feltrini*, *Cynopoecilus* sp. 1, *Cynopoecilus* sp. 2, and *Cynopoecilus* sp.) margins of the Patos Lagoon and the Jacuí River, respectively, agreeing with their allopatric distribution (Figure 1). The exception to this pattern is provided by the eastern populations of *C. melanotaenia*, ex *C. fulgens*, collected in the eastern margin of the Patos Lagoon, in São José do Norte localities 3 and 4.

The pattern of a preferential migration from localities located at the western and/or south of the inferred barriers into those located at the eastern and/or north was supported by the indirect measures of gene flow provided by Lamarc. In fact, whereas migration rates in this direction were on the order of 25.07, rates in the inverse direction were only about 0.01. It is important to emphasize that migration rates in Lamarc are given as a function of M = m/mu, where m is the chance for a lineage to migrate per generation, and mu is the mutation rate per site per generation. So, this analysis revealed that migration constitutes an important source of genetic variability only in the eastern and/or northern populations.

3.2 | Distance analyses

Using GMYC subdivision in 10 cluster, all pairwise comparisons recovered for *COI* showed significant genetic structure and the FST values varied from 0.72 (in the comparison between *C.* sp. 2 and *C.* sp.) to 1.00 (in the comparison between *C.* aff. 2 *nigrovittatus* and

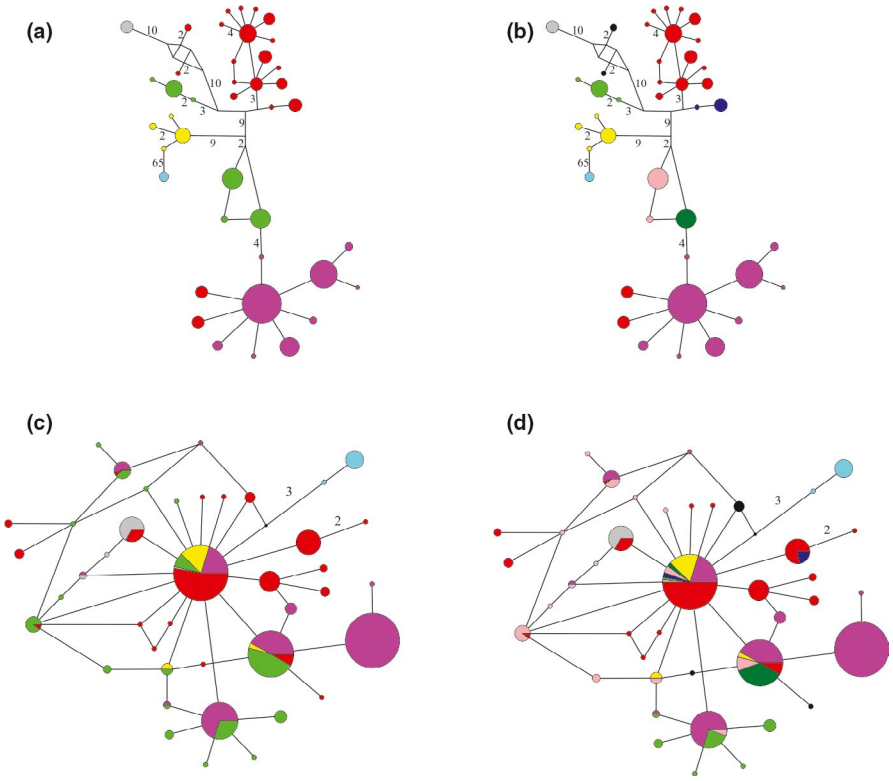

FIGURE 3 Haplotype network constructed with *COI* (a, b) and *RAG1* (c, d) sequences of *Cynopoecilus* emphasizing differences between traditional species boundaries (a, c) and lineages defined after this study (b, d). Number of step-mutations higher than one is in brackets. The size of each circle is proportional to haplotype frequencies, and colors refer to clusters, in accordance with distribution patterns presented in Figure 1

C. notabilis, *C.* aff. 2 *nigrovittatus*, and *C. feltrini* and between *C. notabilis* and *C. feltrini*) (Table 1—values above the diagonal). The *COI* intraspecific mean K2P pairwise distances (Table 1—values on the diagonal) ranged between 0% in *C.* aff. 2 *nigrovittatus*, *C. notabilis*, and *C. feltrini* and 0.9% in *Cynopoecilus* sp. 1. Conversely, mean interspecific pairwise distances (Table 1—values below the diagonal) ranged from 1.2% (as detected between *C.* aff. 1 *nigrovittatus* and *C.* aff. 2 *nigrovittatus*) to values greater than 19.6% (as observed between *C. notabilis* and all other clusters). Considering the presence of a barcoding gap, maximum pairwise intraspecific distances were recovered for *Cynopoecilus* sp. (values of 2.3%) and the minimum pairwise interspecific distances were presented between *C.* aff. 1 *nigrovittatus* and *C.* aff. 2 *nigrovittatus* (values of 1%). Although strictly speaking, this analysis revealed the absence of a strict barcoding gap between GMYC lineages, it is important to highlight that *C.* aff. 1 *nigrovittatus* and *C.* aff. 2 *nigrovittatus* may be just different populations of the same species.

Concerning *RAG1*, all pairwise comparisons between clusters showed significant genetic structure and FST values varied from 0.12 (in the comparison between *C. intimus* and *C.* aff. 1 *nigrovittatus*) to 0.955 (in the comparison between *C. notabilis* and *C. feltrini*) (Table 2). As for this marker, there were several cases of haplotype sharing, mean K2P intra- and interspecific distances were not evaluated.

For ABGD, the number of clusters varied from 13 (for *COI*) to 11 (for the concatenated dataset) when the recursive partition recovered with a prior of 0.0028 is reported. For *COI*, this analysis resulted in further subdivision of clade 3 (*C.* affinis 1 *nigrovittatus*), clade 5 (*C. nigrovittatus*), and clade 8 (*Cynopoecilus* sp. 1), whereas for the concatenated dataset, only clade 3 (*C.* affinis 1 *nigrovittatus*) was further subdivided. When the same prior was evaluated under the initial partition, *COI* recovered 12 clusters, subdividing clusters 3 and 8, whereas the concatenated dataset supported subdivision in eight clusters, joining together clusters 1, 3, and 4 (*C. melanotaenia*, *C.* affinis 1 *nigrovittatus* and *C.* affinis 2 *nigrovittatus*). Although the subdivision of individual populations seems little likely, the grouping of the three closely related and spatially adjacent clusters encompasses an alternative hypothesis for species boundaries.

4 | DISCUSSION

The 275 specimens of *Cynopoecilus* analyzed in the present study encompass 8–10 clusters, very likely corresponding to 8–10 distinct species. This study greatly enhances the currently known fauna of annual fish in the region, not only increasing the number of species of *Cynopoecilus* from six (Costa, 2002b, 2016; Costa et al., 2016; Ferrer et al., 2014) to 8–10, but revealing several cases of previously unrecognized diversity and questioning several taxonomic boundaries. In fact, from the previously known species, only *C. intimus*, *C. notabilis*, and *C. feltrini* revealed reciprocally monophyletic and were validated by this study. *Cynopoecilus fulgens* and *C. nigrovittatus* revealed polyphyletic and were each subdivided into three independent lineages. Furthermore, as individuals of *C. fulgens* from the type locality (Costa, 2002b) nested within *C. melanotaenia*, this study also questioned the taxonomic validity of *C. fulgens* and expanded the distribution of *C. melanotaenia.* In this line of reasoning, our results also revealed several cases where the taxonomic identity of individual populations should be revised. This is especially important because most species of *Cynopoecilus* compose a morphologically homogeneous group of killifish (Costa et al., 2016). This is the case, for example, of population of São José do Norte, locality 3, whose individuals were assigned to *C. melanotaenia*, and not *C. fulgens*; and, individuals from two populations of Viamão previously identified as *C. nigrovittatus*, but grouped here with *Cynopoecilus* sp.

Concerning *COI*, although one of the assumptions of successful application of barcoding approaches [the presence of a barcoding gap between *COI* intra- and interspecific distance ranges (Meyer & Paulay, 2005)] could not be here detected when the 10 clusters are considered, the straightforward application of the second

TABLE 1 *COI* K2P mean intra-cluster (on the diagonal, in bold) and mean inter-lineages distances (below the diagonal), added to pairwise fixation indices (FST) (above the diagonal) between lineages of *Cynopoecilus*

		1	2	3	4	5	6	7	8	9	10
1	*C. melanotaenia*	**0.002**	0.948*	0.889*	0.857*	0.959*	0.987*	0.949*	0.957*	0.951*	0.937*
2	*C. intimus*	0.040	**0.001**	0.966*	0.971*	0.970*	0.990*	0.973*	0.952*	0.971*	0.922*
3	*C.* aff. 1 *nigrovittatus*	0.021	0.030	**0.002**	0.948*	0.985*	0.998*	0.990*	0.970*	0.987*	0.910*
4	*C.* aff. 2 *nigrovittatus*	0.021	0.030	0.012	**0**	0.990*	1.000*	1.000*	0.976*	0.995*	0.908*
5	*C. nigrovittatus*	0.058	0.056	0.056	0.057	**0.001**	0.995*	0.981*	0.947*	0.960*	0.830*
6	*C. notabilis*	0.202	0.198	0.196	0.197	0.205	**0**	1.000*	0.977*	0.998*	0.975*
7	*C. feltrini*	0.060	0.056	0.052	0.051	0.055	0.210	**0**	0.911*	0.991*	0.897*
8	*Cynopoecilus* sp. 1	0.067	0.064	0.054	0.055	0.051	0.207	0.037	**0.009**	0.936*	0.890*
9	*Cynopoecilus* sp. 2	0.053	0.057	0.049	0.050	0.031	0.213	0.049	0.047	**0.002**	0.722*
10	*Cynopoecilus* sp.	0.053	0.061	0.052	0.055	0.038	0.212	0.059	0.055	0.024	**0.006**

Note: The asterisks indicate significant differences ($p < .05$).

TABLE 2 *RAG1* pairwise fixation indices (FST) between lineages of *Cynopoecilus*

		2	3	4	5	6	7	8	9	10
1	*C. melanotaenia*	0.280*	0.194*	0.153*	0.347*	0.702*	0.365*	0.264*	0.379*	0.275*
2	*C. intimus*		0.122*	0.492*	0.520*	0.834*	0.538*	0.418*	0.507*	0.369*
3	*C.* aff. 1 *nigrovittatus*			0.295*	0.341*	0.684*	0.340*	0.160*	0.292*	0.230*
4	*C.* aff. 2 *nigrovittatus*				0.671*	0.882*	0.706*	0.436*	0.644*	0.390*
5	*C. nigrovittatus*					0.909*	0.671*	0.606*	0.675*	0.494*
6	*C. notabilis*						0.955*	0.834*	0.879*	0.709*
7	*C. feltrini*							0.673*	0.744*	0.451*
8	*Cynopoecilus* sp. 1								0.310*	0.207*
9	*Cynopoecilus* sp. 2									0.158*
10	*Cynopoecilus* sp.									

Note: The asterisks indicate significant differences ($p < .05$).

assumption [reciprocal monophyly between species (Kekkonen & Hebert, 2014)] seems to compensate further caveats. In fact, as annual fish usually encompass small and isolated populations (Volcan et al., 2015), important evolutionary consequences can be attained even in short temporal scales, which would be enough to attain reciprocal monophyly (given the strength of genetic drift and the small interference of gene flow), but not to accomplish greater levels of genetic divergences. This highlights the importance of phylogenetic approaches in spite of distance approaches in Rivulidae. Nevertheless, it is also important to consider that the main exception to the application of a barcoding gap is provided by the treatment of *C.* aff. 1 *nigrovittatus* and *C.* aff. 2 *nigrovittatus* as separate lineages. In fact, when these two clusters are merged together, or with *C. melanotaenia*, the minimum interspecific distance raises from 1% to 1.4%.

In spite of this, we confirmed here that DNA barcoding may be a powerful tool for revealing cryptic diversity in Rivulidae, promoting the identification and assisting in the discovery of putative new species. Nevertheless, it is important to emphasize that the subdivision in 8–10 clusters was supported not only by *COI*, but also by *RAG1* and *COI* + *RAG1* datasets, further enhancing confidence in the obtained results. In this sense, although the network reconstructed with *RAG1* revealed a straightforward haplotype sharing between clusters and the phylogenetic tree obtained for this marker did not attain reciprocal monophyly for the individual clusters, this is perfectly explained by the larger fixation time expected for nuclear in comparison with mitochondrial markers in virtue of their larger effective population sizes (Birky, Maruyama, & Fuerst, 1983; Pamilo & Nei, 1988). Even so, *RAG1* also provided important insights into the evolution of *Cynopoecilus*, as the signals of significant population structure among clusters, and the star-like network pattern. In fact, when this last result is considered in the context of the larger coalescence times usually expected for nuclear markers (Templeton, 2006), it suggests an ancient population expansion for the genus, whose signal is already erased for the mitochondrial marker. Thus, the concomitant use of both markers not only corroborated the results provided by *COI* but also added resolution to the evolutionary scenario of *Cynopoecilus*. Although further morphological inspections and other integrative taxonomy approaches (Steinke & Hanner, 2011) are certainly needed in order to confirm that each of these 8–10 clusters really constitutes different species, the independent evolutionary status evidenced here for each them should since now guarantee their individual conservation.

Another interesting result provided by this study is related to the deep split between the most derived species, whose clades are spatially distributed in different sides of the Jacuí River and Patos Lagoon, which seem to have historically acted as effective barriers to gene flow. This major phylogenetic structure encountered in *Cynopoecilus* supports the hypothesis that large water bodies can act as effective barriers to gene flow among populations/species of annual fish, leading to allopatric fragmentation (Bartáková et al., 2013, 2015; García et al., 2012, 2015). This pattern is also consistent with that recently demonstrated for *A. wolterstorffi*, at least in regard to the Patos-Mirim Lagoon System (Garcez et al., 2018). In a finer scale, it was also possible to evidence vicariance as a result of drainage changes related to geomorphological events that affected the Southern Brazil coastal plain during Quaternary (Loureiro & García, 2006; Villwock & Tomazelli, 2007). In this sense, populations previously assigned to *C. fulgens* that inhabit areas in which these recent geomorphological events have occurred confirmed especially prone to present cryptic diversity. Nevertheless, in contrast to what was recently shown for *A. wolterstorffi*, a species that inhabits the same drainage system (Garcez et al., 2018), the phylogenetic tree recovered here for *Cynopoecilus* does not suggests a north-to-south colonization route. In fact, the early offshoot of the genus, that is, *C. notabilis*, does not occur in one of the Northern most distribution areas of the genus.

Although vicariance seems to have accomplished the main diversification mechanism of Rivulidae (Bartáková et al., 2015; Garcez et al., 2018; García et al., 2009, 2012, 2015; Jowers, Cohen, & Downie, 2008; Loureiro & de Sá, 2015; Ponce de León et al., 2014), the distribution of the provisional species of *Cynopoecilus* also suggests the occurrence of isolated and recent dispersal events. This can be evidenced by the signals of population

admixture detected in the locality 4 of São José do Norte (SJN4), which encompasses individuals clustered with *C. melanotaenia* and with *Cynopoecilus* sp., and by the single genetically homogeneous population of *C. melanotaenia* (initially assigned to *C. fulgens*) sampled at the East margin of the Lagoon (locality 3 of São José do Norte—SJN3). Both these populations are separated from the other populations of *C. melanotaenia* by the Patos Lagoon estuary. For SJN4, there seems to have occurred an admixture or secondary contact, as that encountered between *A. charrua* Costa & Cheffe, 2001 and *A. reicherti* Loureiro & García, 2004 (García et al., 2009) and between two clusters of *A. wolterstorffi* (Garcez et al., 2018). In both cases, the lowlands of the Patos-Mirim drainage system near the Atlantic coast were considered as potential contact areas (Garcez et al., 2018; García et al., 2009, 2015), in accordance with the scenario depicted here. Nevertheless, the fact that there are populations of *C. melanotaenia* inhabiting both margins of the Patos Lagoon suggests the occurrence of dispersal events across the Lagoon, which sounds quite surprising. Since it was recently demonstrated that aquatic birds can transport embryos of annual fish in their digestive traits (Silva et al., 2019), we hypothesize here birds may have been involved in such wide-range dispersal events. Interestingly, nevertheless, migration rates are quite larger in the west/south to east/north direction than in the reverse, imposing a sense of contingency or the need for other explanatory events. Although evidence of a paleochannel in the same area of the putative dispersal (Weschenfelder, Corrêa, Aliotta, & Baitelli, 2010) may provide opportunities for differential gene flow, the geographical and evolutionary distribution of the migration signals (which affect geographically adjacent populations of a single lineage of *C. melanotaenia*) suggest they derive from a single dispersal event across the Patos Lagoon.

5 | CONCLUSION

Our study showed that DNA barcoding is a highly reliable tool to delimit *Cynopoecilus* species or lineages, especially if complemented by the concomitant use of a nuclear marker. Examined individuals represent 8–10 well-supported clusters, which comprise different populations of the six previously described species. In this sense, one species was considered invalid (*C. fulgens*), whereas other three-five undescribed putative new species were presented (*Cynopoecilus* sp., *Cynopoecilus* sp. 1, *Cynopoecilus* sp. 2, and possibly *C.* aff. 1 *nigrovittatus* and *C.* aff. 2 *nigrovittatus*). Some of these cryptic species probably diverged as a result of vicariant events, promoted by large water bodies like the Jacuí River and the Patos Lagoon, although isolated large-scale dispersal events across the last barrier could be also evidenced.

This study certainly adds to the mounting body of evidence indicating that annual fish diversity is currently underestimated (Garcez et al., 2018; Volcan et al., 2015) and that there is an urgent need of further research and conservation efforts in the group (Volcan & Lanés, 2018; Volcan et al., 2015). The current lack of knowledge about the actual biodiversity levels limits our ability to quantify the true extent of freshwater biodiversity, as well as our ability to alleviate potential threats (Dudgeon et al., 2006). Thus, the delimitation of putative new species and the refinement of the knowledge about distribution areas, as provided here, not only increases objectivity, but also enhances further works with these taxonomic groups (Kekkonen & Hebert, 2014).

ACKNOWLEDGEMENTS

We are grateful to Luiz Malabarba and Juliano Ferrer who kindly provided tissues from *C. notabilis*, *C. melanotaenia*, *Cynopoecilus* sp., and *Cynopoecilus* sp. 2. We also thank to Marcelo Loureiro, for providing specimens of *C. melanotaenia* from Uruguay, and for reviewing this article. This study was funded by Fundação Grupo Boticário de Proteção à Natureza as part of the "Padrões micro e macroevolutivos em peixes anuais de *Cynopoecilus* e *Austrolebias* (Cyprinodontiformes: Rivulidae) ao longo do Sistema de Drenagens Patos-Mirim: um enfoque comparativo com aplicações para a conservação—1090_20171" project. Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES) —Finance Code 001 provided fellowships to Daiana Kaster Garcez and Murilo de Oliveira Fernandes. All the collections were authorized by the Brazilian Ministério do Meio Ambiente (MMA), in the form of the Sistema de Autorização e Informação em Biodiversidade (SISBIO) (process number 55651-3). This study was also approved by the Ethics Comittee on Animal Use of the Universidade Federal do Rio Grande (process number 23116.008163/2015-23).

ORCID

Daiana Kaster Garcez https://orcid.org/0000-0003-4526-9647
Murilo de Oliveira Fernandes https://orcid.org/0000-0003-2030-4119
Gabrielli da Rosa Ozório https://orcid.org/0000-0002-7173-0751
Matheus Vieira Volcan https://orcid.org/0000-0001-5846-3786
Lizandra Jaqueline Robe https://orcid.org/0000-0001-8506-9143

REFERENCES

Bandelt, H. J., Forster, P., & Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, *16*(1), 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036

Barbosa, C., Garcez, D. K., Volcan, M. V., & Robe, L. J. (2020). Evidences for genetic differentiation within the highly endemic and endangered annual fish *Austrolebias nigrofasciatus* (Cyprinodontiformes: Rivulidae). *Journal of Fish Biology*, *96*, 154–267. https://doi.org/10.1111/jfb.14201

Bartáková, V., Reichard, M., Blažek, R., Polačik, M., & Bryja, J. (2015). Terrestrial fishes: rivers are barriers to gene flow in annual fishes from the African savanna. *Journal of Biogeography*, *42*, 1832–1844. https://doi.org/10.1111/jbi.12567

Bartáková, V., Reichard, M., Janko, K., Polačik, M., Blažek, R., Reichwald, K., … Bryja, J. (2013). Strong population genetic structuring in an annual fish, *Nothobranchius furzeri*, suggests multiple savannah refugia in southern Mozambique. *BMC Evolutionary Biology*, *13*(196), https://doi.org/10.1186/1471-2148-13-196

Birky, C. W., Maruyama, T., & Fuerst, P. (1983). An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplasts, and some results. *Genetics*, *103*(3), 513–527.

Costa, W. J. E. M. (2002a). *Peixes Anuais Brasileiros: Diversidade e Conservação*. Curitiba, PR: UFPR.

Costa, W. J. E. M. (2002b). The annual fish genus *Cynopoecilus* (Cyprinodontiformes: Rivulidae): taxonomic revision, with descriptions of four new species. *Ichthyological Exploration of Freshwaters*, *13*(1), 11–24.

Costa, W. J. E. M. (2008). *Catalog of Aplocheiloid killifishes of the world*. Rio de Janeiro, RJ: UFRJ.

Costa, W. J. E. M. (2016). Comparative morphology and classification of South American cynopoeciline killifishes (Cyprinodontiformes: Aplocheilidae), with notes on family-group names used for aplocheiloids. *Vertebrate Zoology*, *66*(2), 125–140.

Costa, W. J. E. M., & Amorim, P. F. (2011). A new annual killifish species of the *Hypsolebias flavicaudatus* complex from the São Francisco River basin, Brazilian Caatinga (Cyprinodontiformes: Rivulidae). *Vertebrate Zoology*, *61*(1), 99–104.

Costa, W. J. E. M., Amorim, P. F., & Aranha, G. M. (2014). Species limits and DNA barcodes in *Nematolebias*, a genus of seasonal killifishes threatened with extinction from the Atlantic Forest of south-eastern Brazil, with description of a new species (Teleostei: Rivulidae). *Ichthyological Exploration of Freshwaters*, *24*(3), 225–236.

Costa, W. J. E. M., Amorim, P. F., & Mattos, J. L. O. (2016). A new species of inseminating seasonal killifish of the *Cynopoecilus melanotaenia* complex from southern Brazil (Cyprinodontiformes: Rivulidae). *Biodiversity Data Journal*, *4*, e6888. https://doi.org/10.3897/BDJ.4.e6888

Costa, W. J. E. M., & Cheffe, M. M. (2001). Three new annual fishes of the genus Austrolebias from the Laguna dos Patos system, Southern Brazil, and a re-description of A. adloffi (Ahl) (Cyprinodontiformes: Rivulidae). *Comunicações do Museu de Ciência e Tecnologia*, *PUCRS*, *Série Zoologia*, *Porto Alegre*, *14*(2), 179–200.

Drummond, A. J., Suchard, M. A., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*, *29*(8), 1969–1973. https://doi.org/10.1093/molbev/mss075

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z.-I., Knowler, D. J., Lévêque, C., … Sullivan, C. A. (2006). Freshwater biodiversity: Importance, threats, status and conservation challenges. *Biological Reviews*, *81*(2), 163–182. https://doi.org/10.1017/S1464793105006950

Excoffier, L., & Lischer, H. E. L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, *10*(3), 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Ferrer, J., Wingert, J. M., & Malabarba, L. R. (2014). Description of a new species and phylogenetic analysis of the subtribe Cynopoecilina, including continuous characters without discretization (Cyprinodontiformes: Rivulidae). *Zoological Journal of the Linnean Society*, *172*(4), 846–866. https://doi.org/10.1111/zoj.12190

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*(5), 294–299.

Funk, D. J., & Omland, K. E. (2003). Species-level paraphyly and polyphyly: Frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics*, *34*, 397–423. https://doi.org/10.1146/annurev.ecolsys.34.011802.132421

Garcez, D. K., Barbosa, C., Loureiro, M., Volcan, M. V., Loebmann, D., Quintela, F. M., & Robe, L. J. (2018). Phylogeography of the critically endangered neotropical annual fish, *Austrolebias wolterstorffi* (Cyprinodontiformes: Aplocheilidae): genetic and morphometric evidence of a new species complex. *Environmental Biology of Fishes*, *101*(10), 1503–1515. https://doi.org/10.1007/s10641-018-0795-2

García, G., Gutiérrez, V., Ríos, N., & de Sá, R. O. (2015). Comparative phylogeographic patterns in *Austrolebias* from different South American Basins. In N. Berois, G. García, & R. O. de Sá (Eds.), *Annual fishes: Life history strategy, diversity, and evolution* (pp. 259–279). Boca Raton, FL: Press Taylor & Francis group.

García, G., Gutiérrez, V., Ríos, N., Serra, S., Calviño, P., Duarte, A., & Loureiro, M. (2019). Contrasting morphology with population genetics approach: An insight to revision of the Neotropical annual fish "*Austrolebias robustus*" species group based on a taxonomic integrative framework. *Journal of Zoological Systematics and Evolutionary Research*, *57*(3), 1–15. https://doi.org/10.1111/jzs.12268

García, G., Gutiérrez, V., Vergara, J., Calviño, P., Duarte, A., & Loureiro, M. (2012). Patterns of population differentiation in annual killifishes from the Paraná-Uruguay-La Plata basin: The role of vicariance and dispersal. *Journal of Biogeography*, *39*(9), 1707–1719. https://doi.org/10.1111/j.1365-2699.2012.02722.x

García, G., Loureiro, M., Berois, N., Arezo, M. J., Casanova, G., Clivio, G., & Olivera, A. (2009). Pattern of differentiation in the annual killifish genus *Austrolebias* (Cyprinodontiformes; Rivulidae) from a biosphere reserve site in South America: A multidisciplinary approach. *Biological Journal of the Linnean Society*, *98*(3), 620–635. https://doi.org/10.1111/j.1095-8312.2009.01303.x

Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(41), 14812–14817. https://doi.org/10.1073/pnas.0406166101

Hebert, P. D. N., Ratnasingham, S., & de Waard, J. R. (2003). Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society B: Biological Sciences*, *270*(Suppl_1), S96–S99. https://doi.org/10.1098/rsbl.2003.0025

ICMBio, Instituto Chico Mendes de Conservação da Biodiversidade. (2018). Sumário executivo do plano de ação nacional para a conservação dos peixes Rivulídeos ameaçados de extinção. Brasília. Retrieved from http://www.icmbio.gov.br/portal/biodiversidade/fauna-brasileira/plano-de-acao/2833-plano-de-acao-nacional-para-a-conservacao-dos-rivulideos.html. Accessed 20 January 2019.

Jowers, M. J., Cohen, B. L., & Downie, J. R. (2008). The cyprinodont fish *Rivulus* (Aplocheiloidei: Rivulidae) in Trinidad and Tobago: Molecular evidence for marine dispersal, genetic isolation and local differentiation. *Journal of Zoological Systematics and Evolutionary Research*, *46*(1), 48–55. https://doi.org/10.1111/j.1439-0469.2007.00422.x

Kekkonen, M., & Hebert, P. D. N. (2014). DNA barcode-based delineation of putative species: Efficient start for taxonomic workflows. *Molecular Ecology Resources*, *14*(4), 706–715. https://doi.org/10.1111/1755-0998.12233

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*(2), 111–120. https://doi.org/10.1007/BF01731581

Kiontke, K. C., Félix, M. A., Ailion, M., Rockman, M. V., Braendle, C., Pénigault, J. B., & Fitch, D. H. (2011). A phylogeny and molecular barcodes for Caenorhabditis, with numerous new species from rotting fruits. *BMC Evolutionary Biology*, *11*(339), https://doi.org/10.1186/1471-2148-11-339

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Kuhner, M. K. (2006). LAMARC 2.0: maximum likelihood and Bayesian estimation of population parameters. *Bioinformatics*, *22*(6), 768–770. https://doi.org/10.1093/bioinformatics/btk051

Librado, P., & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, *25*(11), 1451–1452. https://doi.org/10.1093/bioinformatics/btp187

Loureiro, M., & de Sá, R. O. (2015). Diversity of Aplocheiloidei. In N. Berois, G. García, & R. O. de Sá (Eds.), *Annual fishes: Life history strategy, diversity, and evolution* (pp. 3–31). Boca Raton, FL: Press Taylor & Francis group.

Loureiro, M., de Sá, R., Serra, S. W., Alonso, F., Lanés, L. E. K., Volcan, M. V., … García, G. (2018). Review of the family Rivulidae (Cyprinodontiformes, Aplocheiloidei) and a molecular and morphological phylogeny of the annual fish genus *Austrolebias* Costa 1998. *Neotropical Ichthyology*, 16(3), e180007. https://doi.org/10.1590/1982-0224-20180007

Loureiro, M., & García, G. (2004). Cynolebias reicherti a new annual fish (Rivulidae:Cynolebiatinae) from southern Laguna Merim basin. *Acta Zoológica Lilloana*, *48*, 13–25.

Loureiro, M., & García, G. (2006). Transgresiones y regresiones marinas en la costa Atlántica y lagunas costeras de Uruguay: Efectos sobre los peces continentales. In R. Menafra, L. Rodríguez-Gallego, F. Scarabino, & D. Conde (Eds.), *Bases para la conservación y el manejo de la costa uruguaya* (pp. 545–556). Uruguay: Vida Silvestre.

Machado, S., Junges dos Santos, J. P., Fonseca, P. M., Bolzan, A. R., David, J., Loreto, E. L. D. S., … Robe, L. J. (2017). Neotropical mycophagous drosophilids (Diptera: Drosophilidae): DNA barcoding as a way of overcoming the taxonomic impediment. *Insect Conservation and Diversity*, *10*(3), 271–281. https://doi.org/10.1111/icad.12223

Meyer, C. P., & Paulay, G. (2005). DNA barcoding: Error rates based on comprehensive sampling. *PLoS Biology*, *3*(12), e422. https://doi.org/10.1371/journal.pbio.0030422

Monaghan, M. T., Wild, R., Elliot, M., Fujisawa, T., Balke, M., Inward, D. J., … Vogler, A. P. (2009). Accelerated species inventory on Madagascar using coalescent-based models of species delineation. *Systematic Biology*, *58*(3), 298–311. https://doi.org/10.1093/sysbio/syp027

Moritz, C., & Cicero, C. (2004). DNA barcode: Promise and pitfalls. *PLoS Biology*, *02*, 1529–1531. https://doi.org/10.1371/journal.pbio.0020354

Murphy, W. J., Thomerson, J. E., & Collier, G. E. (1999). Phylogeny of the Neotropical Killifish Family Rivulidae (Cyprinodontiformes, Aplocheiloidei) inferred from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, *13*(2), 289–301. https://doi.org/10.1006/mpev.1999.0656

Near, T. J., Eytan, R. I., Dornburg, A., Kuhn, K. L., Moore, J. A., Davis, M. P., … Smith, W. L. (2012). Resolution of ray-finned fish phylogeny and timing of diversification. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(34), 13698–13703. https://doi.org/10.1073/pnas.1206625109

Packer, L., Gibbs, J., Sheffield, C., & Hanner, R. (2009). DNA Barcode and the mediocrity of morphology. *Molecular Ecology Resources*, *9*, 42–50. https://doi.org/10.1111/j.1755-0998.2009.02631.x

Pamilo, P., & Nei, M. (1988). Relationships between gene trees and species trees. *Molecular Biology and Evolution*, *5*(5), 568–583. https://doi.org/10.1093/oxfordjournals.molbev.a040517

Pfeiler, E. (2018). DNA barcoding and taxonomic challenges in describing new putative species: Examples from Sootywing and Cloudywing Butterflies (Lepidoptera: Hesperiidae). *Diversity*, *10*(4), 111–125. https://doi.org/10.3390/d10040111

Ponce de León, J. L., León, G., Rodríguez, R., Metcalfe, C. J., Hernández, D., Casane, D., & García-Machado, E. (2014). Phylogeography of Cuban *Rivulus*: Evidence for allopatric speciation and secondary dispersal across a marine barrier. *Molecular Phylogenetics and Evolution*, *79*, 404–414. https://doi.org/10.1016/j.ympev.2014.07.007

Pons, J., Barraclough, T., Gomez-Zurita, J., Cardoso, A., Duran, D., Hazell, S., … Vogler, A. (2006). Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology*, *55*(4), 595–609. https://doi.org/10.1080/10635150600852011

Posada, D. (2008). jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution*, *25*(7), 1253–1256. https://doi.org/10.1093/molbev/msn083

Puillandre, N., Lambert, A., Brouillet, S., & Achaz, G. (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology*, *21*(8), 1864–1877. https://doi.org/10.1111/j.1365-294X.2011.05239.x

Rambaut, A., & Drummond, A. J. (2009). FigTree, v1.4.3. Retrieved from http://tree.bio.ed.ac.uk/software/figtree/. Accessed 18 January 2019.

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard, M. A. (2018). Tracer v1.7. http://tree.bio.ed.ac.uk/software/tracer/. Accessed 20 January 2019.

Regan, C. T. (1912a). A revision of the poeciliid fishes of the genera *Rivulus*, *Pterolebias*, and *Cynolebias*. *The Annals and Magazine of Natural History; Zoology, Botany, and Geology*, *10*(8), 494–508.

Regan, C. T. (1912b). Sexual differences in the poeciliid fishes of the genus *Cynolebias*. *The Annals and Magazine of Natural History; Zoology, Botany, and Geology*, *10*(8), 641–642.

Reichard, M. (2015). The evolutionary ecology of african annual fishes. In N. Berois, G. García, & R. O. de Sá (Eds.), *Annual fishes: Life history strategy, diversity, and evolution* (pp. 133–158). Boca Raton, FL: Press Taylor & Francis group.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., … Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*(3), 1–4. https://doi.org/10.1093/sysbio/sys029

Sambrook, J., Fritschi, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual*. New York, NY: Cold Spring Harbor Laboratory Press.

Schwarz, G. (1978). Estimating the dimension of a model. *The Annals of Statistics*, *6*(2), 461–464. https://doi.org/10.1214/aos/1176344136

SEMA, Secretaria Estadual de Meio Ambiente. (2014). Reavaliação da Lista das Espécies da Fauna Silvestre Ameaçadas de Extinção no Rio Grande do Sul. http://www.liv.fzb.rs.gov.br/livcpl/?idmodulo=1and id_uf=23. Accessed 20 January 2019.

Silva, G. G., Weber, V., Green, A. J., Hoffmann, P., Silva, V. S., Lanés, L. E. K., … Maltchik, L. (2019). Killifish eggs can disperse via gut passage through waterfowl. *Ecology*, *100*(11), e02774. https://doi.org/10.1002/ecy.2774

Song, H., Buhay, J. E., Whiting, M. F., & Crandall, K. A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences*, *105*(36), 13486–13491. https://doi.org/10.1073/pnas.0803076105

Staden, R. (1996). The Staden sequence analysis package. *Molecular Biotechnology*, *5*, 233–241. https://doi.org/10.1007/BF02900361

Steinke, D., & Hanner, R. (2011). The FISH-BOL collaborators' protocol. *Mitochondrial DNA*, *22*(1), 10–14. https://doi.org/10.3109/19401736.2010.536538

Templeton, A. R. (2006). *Population genetics and microevolutionary theory*. Hoboken, NJ: John Wiley & Sons.

Villwock, J. A., & Tomazelli, L. J. (2007). Planície Costeira do Rio Grande do Sul: gênese e paisagem atual. In F. G. Becker, R. A. Ramos, & L. A. Moura (Eds.), *Biodiversidade. Regiões da Lagoa do Casamento e dos Butiazais de Tapes, planície costeira do Rio Grande do Sul*. (pp. 20–33). Brasília: MMA/SBF.

Volcan, M. V., & Lanés, L. E. K. (2018). Brazilian killifishes risk extinction. *Science*, *361*(6400), 340–341. https://doi.org/10.1126/science.aau5930

Volcan, M. V., Lanés, L. E. K., Gonçalves, A. C., & Guadagnin, D. L. (2015). Annual fishes (Rivulidae) from Southern Brazil: A broad-scale assessment of their diversity and conservation. In N. Berois, G. García, & R. O. de Sá (Eds.), *Annual Fishes: Life History Strategy, Diversity, and Evolution* (pp. 185–203). Boca Raton, FL: Press Taylor & Francis group.

Weschenfelder, J., Corrêa, I. C. S., Aliotta, S., & Baitelli, R. (2010). Paleochannels related to late quaternary sea-level changes in Southern Brazil. *Brazilian Journal of Oceanography*, *58*(2), 35–44. https://doi.org/10.1590/S1679-87592010000600005

Witt, J. D., Threloff, D. L., & Hebert, P. D. (2006). DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: Implications for desert spring conservation. *Molecular Ecology*, *15*(10), 3073–3082. https://doi.org/10.1111/j.1365-294X.2006.02999.x

Zimmermann, B. L., Campos-Filho, I. S., Deprá, M., & Araujo, P. B. (2015). Taxonomy and molecular phylogeny of the Neotropical genus *Atlantoscia* (Oniscidea, Philosciidae): DNA barcoding and description of two new species. *Zoological Journal of the Linnean Society*, *174*(4), 702–717. https://doi.org/10.1111/zoj.12256

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. List of sampling localities for each of the major lineages recovered in this study, with their respective identification codes and geographical coordinates.

Alignment S1. *COI* alignment.

Alignment S2. *RAG1* alignment.

How to cite this article: Kaster Garcez D, deOliveira Fernandes M, daRosa Ozório G, Vieira Volcan M, Robe LJ. Phylogenetic structure of Neotropical annual fish of the genus *Cynopoecilus* (Cyprinodontiformes: Rivulidae), with an assessment of taxonomic implications. *J Zool Syst Evol Res*. 2020;00:1–12. https://doi.org/10.1111/jzs.12389