



Universidade Federal do Rio Grande
Instituto de Ciências Biológicas
Pós-graduação em Biologia de
Ambientes Aquáticos Continentais



**Taxonomia e padrões micro e macroevolutivos em
peixes anuais dos gêneros *Cynopoecilus* e
Austrolebias (Cyprinodontiformes: Rivulidae) com
aplicações para a conservação**

Daiana Kaster Garcez

Orientadora: Prof^a Dr^a Lizandra Jaqueline Robe

Rio Grande
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Aluna: Daiana Kaster Garcez

Orientadora: Prof^a Dr^a Lizandra Jaqueline Robe

Tese apresentada ao Programa de Pós-graduação em Biologia de Ambientes Aquáticos Continentais como requisito parcial para a obtenção do título de Doutora em Biologia de Ambientes Aquáticos Continentais.

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**ATA DE DEFESA DE TESE DE DOUTORADO EM BIOLOGIA DE AMBIENTES
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Às 14:00 hs (quatorze horas) do dia 30 (trinta) do mês de abril de 2021 (dois mil e vinte e um), via Webconferência (endereço eletrônico: (<https://meet.google.com/xnu-gsdo-qfk>) reuniram-se docentes, discentes e comunidade em geral, para a Defesa Pública da Tese de Doutorado da acadêmica **Daiana Kaster Garcez**. A Tese intitulada **"Taxonomia e padrões micro e macroevolutivos em peixes anuais dos gêneros *Cynopoecilus* e *Austrolebias* (Cyprinodontiformes: Rivulidae) com aplicações para a conservação"** foi avaliada pela Banca Examinadora composta pela Prof^a. Dra. Lizandra Jaqueline Robe (Orientadora), Prof^o Dr. Alexandre Miranda Garcia (FURG), Prof^o Dr. Luis Esteban Krause Lanés (IPPampa) e Prof^o Dr. Marcelo Loureiro Barrella (UDELAR). Após a defesa e arguição pública, a Banca Examinadora reuniu-se, para deliberação final, e considerou a acadêmica **APROVADA**. Desta forma, a acadêmica concluiu mais uma das etapas necessárias para a obtenção do grau de **DOUTORA EM BIOLOGIA DE AMBIENTES AQUÁTICOS CONTINENTAIS**. Nada mais havendo a tratar, às 17:50 hs (17 horas e cinquenta minutos) foi lavrada a presente ata, que lida e aprovada, foi assinada pelos membros da Banca Examinadora, pela Acadêmica e pelo Coordenador do Curso.

Prof^a Dra. Lizandra Jaqueline Robe

Prof^o Dr. Alexandre Miranda Garcia

Prof^o Dr. Luis Esteban Krause Lanés

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Daiana

Profº Dr. Rogério Tubino Vianna
Coordenador do Curso

Dedico esta tese aos que sempre estiveram ao meu lado me apoiando nessa jornada
Minha mãe Dóris Helena (*in memoriam*), meu pai Nilson, meus irmãos Franklin e Geferson e
meu marido Geison

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RESUMO

A biodiversidade do nosso planeta está diminuindo drasticamente como consequência das ações humanas, e alguns táxons são particularmente suscetíveis a estas ameaças. Os peixes anuais destacam-se, dentro deste contexto, como um dos grupos de vertebrados mais ameaçados. Estes peixes estão sujeitos à ação de um conjunto de condições evolutivas, demográficas e metabólicas que podem resultar na rápida divergência entre populações. Alguns destes mecanismos, acrescidos da perda, degradação e fragmentação das áreas úmidas explicam a maior vulnerabilidade destas espécies. No Neotrópico, peixes anuais dos gêneros *Cynopoecilus* e *Austrolebias* se destacam por apresentarem muitas espécies endêmicas do Sistema Lagunar Patos-Mirim (SLPM) ameaçadas de extinção. Esta área é considerada um *hotspot* de diversidade de peixes anuais, embora regiões estratégicas para a conservação destas espécies ainda não tenham sido delimitadas. Aliado a isso, divergências sobre o status taxonômico das espécies de *Cynopoecilus* e o desconhecimento da sua história evolutiva destacam a importância de estudos filogenéticos e filogeográficos voltados para estas espécies. Assim, a presente Tese tem como objetivo auxiliar na delimitação de estratégias de conservação *in situ* de peixes anuais dos gêneros *Cynopoecilus* e *Austrolebias*, através do esclarecimento de questões taxonômicas, micro e macroevolutivas. Para isso, foram realizadas análises moleculares, morfológicas e de distribuição de endemismo, simpatria e riqueza. No Capítulo 1, a técnica de DNA barcode e análises filogenéticas e de distância detectaram a subdivisão de *Cynopoecilus* em 8–10 grupos, que compreendem as seis espécies descritas anteriormente e sugerem pelo menos 3–5 novas espécies putativas. Em geral, os resultados destacam a necessidade de estratégias de conservação independentes para cada grupo. No Capítulo 2, *Cynopoecilus* sp. n., uma nova espécie do complexo *C. melanotaenia*, é descrita com base em indivíduos coletados em poças temporárias localizadas nas bacias do rio Guaíba e do rio Jacuí, sistema Laguna dos Patos, Rio Grande do Sul, Brasil. Com os resultados encontrados para os marcadores moleculares, sugere-se uma rota de colonização norte-sul para o complexo *C. melanotaenia*–*Cynopoecilus* sp. n. na margem esquerda da Laguna dos Patos, que ocorreu durante os últimos 150 mil anos. Foi evidenciada também a ação do rio Jacuí como barreira entre *Cynopoecilus* sp. n. e *C. nigrovittatus*, que são registradas nas margens opostas deste rio. Por fim, no Capítulo 3, padrões de riqueza de linhagens mostraram uma distribuição heterogênea de peixes anuais ao longo do SLPM, com sete áreas apresentando registros para quatro ou mais linhagens. A aplicação de novos cálculos que levam em conta o número de populações, a diversidade genética e o posicionamento filogenético permitiram elencar as espécies avaliadas por ordem de prioridade de conservação, o que foi utilizado, por fim, para avaliar a ordem de prioridade de conservação dos diferentes micro-*hotspots* de peixes anuais.

Pode-se dizer, por fim, que esta Tese apresenta resultados importantes no âmbito da conservação de peixes anuais ao longo do SLPM, destacando a importância de estudos taxonômico-evolutivos na avaliação das unidades a serem conservadas, tanto no âmbito biológico quanto geográfico.

Palavras-chave: Diversidade críptica; DNA barcode; Filogenia; Filogeografia; *Hotspots*; *Killifish*.

ABSTRACT

Biodiversity in our planet is drastically decreasing as a consequence of human actions, and some taxons are particularly susceptible to these threats. Annual fish detach in this context as one of the more threaten groups of vertebrates. These species are subject to the action of a set of evolutionary, demographic, and metabolic conditions that can result in rapid divergence between populations. Some of these mechanisms, in addition to the loss, degradation and fragmentation of wetlands explain the higher vulnerability of these species. In the Neotropics, annual fish of the genera *Cynopoecilus* and *Austrolebias* stand out for presenting many species endemic to the Patos-Mirim Lagoon System (PMLS) threatened with extinction. This area is considered a hotspot for the diversity of annual fish, although no strategic regions for the conservation of these species were yet defined. In addition, divergences on the taxonomic status of *Cynopoecilus* species and unfamiliarity with their evolutionary history highlight the importance of phylogenetic and phylogeographic studies focused on these species. Thus, this Thesis aims to assist in the delimitation of *in situ* conservation strategies for annual fish of the genera *Cynopoecilus* and *Austrolebias*, by clarifying taxonomic, micro and macroevolutionary issues. For this, molecular, morphological and distribution analyzes of endemism, sympatry and richness were carried out. In Chapter 1, DNA barcoding technique and phylogenetic and distance analyzes detected the subdivision of *Cynopoecilus* into 8–10 groups, which comprise the six species previously described and suggest at least 3–5 putative new species. In general, the results highlight the need for independent conservation strategies for each group. In Chapter 2, *Cynopoecilus* sp. n., a new species of the *C. melanotaenia* complex, is described based on individuals collected in temporary pools located in the Guaíba River and Jacuí River basins, Laguna dos Patos system, Rio Grande do Sul, Brazil. With the results found for the molecular markers, a north-south colonization route was suggested for the *C. melanotaenia*–*Cynopoecilus* sp. n. complex on the left bank of the Patos Lagoon, which occurred during the last 150 thousand years. The action of the Jacuí River as a barrier between *Cynopoecilus* sp. n. and *C. nigrovittatus*, which are recorded on the opposite banks of this river was also evidenced. Finally, in Chapter 3, patterns of lineage richness showed a heterogeneous distribution of annual fish over the sampling area PMLS, with seven areas showing records for four or more lineages. The use of new metrics considering the number of populations together with measures of genetic diversity and phylogenetic positioning allowed to list the evaluated species in order of priority of conservation, which was finally employed to evaluate the priority order of conservation for the different micro-hotspots of annual fish. Thus, this Thesis presents important results concerning the conservation of annual fish along the PMLS, highlighting the importance of taxonomic-

evolutionary studies in the evaluation of the biologic and geographic units to be conserved.

Key-words: Cryptic diversity; DNA barcoding; Phylogeny; Phylogeography; Hotspots; Killifish.

APRESENTAÇÃO

A presente Tese resulta do projeto de pesquisa que desenvolvi durante o curso de Doutorado em Biologia de Ambientes Aquáticos Continentais (PPGBAC), na Universidade Federal do Rio Grande (FURG). O objetivo central da minha tese foi auxiliar na delimitação de estratégias de conservação de peixes anuais dos gêneros *Cynopoecilus* e *Austrolebias* ao longo do sistema lagunar Patos-Mirim (SLPM). Para tanto, busquei recuperar a estrutura filogenética de peixes anuais do gênero *Cynopoecilus* e avaliar suas implicações no âmbito da taxonomia e conservação; apresentar a história da colonização de *Cynopoecilus* ao longo da margem oeste do sistema lagunar Patos-Mirim; e descrever uma nova espécie; demarcar *hotspots* com maiores níveis de endemismo, simpatria e riqueza de peixes anuais ao longo do SLPM, avaliando as prioridades de conservação das linhagens genéticas e suas áreas de ocorrência. Assim, pude avaliar questões taxonômicas e micro e macroevolutivas destes peixes anuais.

Na Introdução Geral apresento uma descrição mais detalhada do meu tema de estudo, bem como dos ambientes e das espécies estudadas, além das ferramentas utilizadas. Os resultados, separados em três artigos, estão apresentados nos capítulos que compõem a Tese. O primeiro artigo, que aborda a estrutura filogenética de peixes anuais do gênero *Cynopoecilus* e avalia suas implicações taxonômicas e conservacionistas, foi publicado no **Journal of Zoological Systematics and Evolutionary Research** e está apresentado no Capítulo 1. O segundo artigo, que compõe o Capítulo 2, apresenta a história da colonização de *Cynopoecilus* ao longo da margem oeste do Sistema Lagunar Patos-Mirim e a descrição de uma nova espécie. Este artigo será submetido para a revista **Journal of Fish Biology**. O terceiro artigo versa sobre a identificação de *hotspots* de peixes anuais ao longo do SLPM e áreas adjacentes. Este artigo foi submetido para a revista **Biodiversity and Conservation**, e está apresentado no Capítulo 3. Após, apresento as considerações finais e perspectivas abertas pelo estudo. Finalizo a Tese apresentando algumas publicações extras produzidas durante o período do doutorado (Anexo 1). Cada capítulo está escrito de acordo com as normas das respectivas revistas para as quais os artigos foram/serão submetidos e tais normas estão disponíveis nos links ao final desta sessão. A Introdução Geral e as Considerações finais e perspectivas estão apresentadas seguindo as normas da ABNT.

Conduzi as atividades de pesquisa deste projeto contando com a colaboração de alguns pesquisadores e instituições. Além do Laboratório de Genética do Instituto de Ciências Biológicas da FURG, onde estive sediada durante o meu mestrado e doutorado, também conduzi análises no Instituto Pró-Pampa, em Pelotas.

As amostragens realizadas no Brasil foram feitas com autorização do Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio/MMA) sob o número 55651-1. A utilização dos animais para fins de pesquisa foi certificada pela Comissão de Ética em Uso Animal – CEUA, da FURG, sob o número P036/2015 (Anexo 2). A aquisição de amostras de exemplares do Uruguai foi possibilitada pela parceria com pesquisadores da Universidad de la República Uruguay.

Links para normas utilizadas na elaboração de cada capítulo

Capítulo 1: *Journal of Zoological Systematics and Evolutionary Research*

<https://onlinelibrary.wiley.com/page/journal/14390469/homepage/forauthors.html>

Capítulo 2: *Journal of Fish Biology*

<https://onlinelibrary.wiley.com/page/journal/10958649/homepage/forauthors.html>

Capítulo 3: *Biodiversity and Conservation*

<https://www.springer.com/journal/10531/submission-guidelines>

SUMÁRIO

AGRADECIMENTOS	vii
RESUMO.....	ix
ABSTRACT	xi
APRESENTAÇÃO.....	xiii
LISTA DE FIGURAS.....	xviii
LISTA DE TABELAS.....	xxi
INTRODUÇÃO GERAL	23
Áreas úmidas e peixes anuais	26
Área de estudo	32
Ferramentas de estudo	33
<i>Taxonomia integrativa: combinando dados morfológicos e moleculares</i>	33
<i>Hotspots de biodiversidade</i>	34
OBJETIVO GERAL	35
Objetivos específicos	35
HIPÓTESES	36
REFERÊNCIAS BIBLIOGRÁFICAS	36
CAPÍTULO 1.....	43
Phylogenetic structure of Neotropical annual fish of the genus <i>Cynopoecilus</i> (Cyprinodontiformes: Rivulidae), with an assessment of taxonomic implications.....	43
1 INTRODUCTION	44
2 MATERIAL AND METHODS.....	45
2.2 DNA manipulation.....	46
2.3 Matrix construction	46
2.4 Phylogenetic and coalescence analyses	47
2.5 Distance analyses	47
2.6 Migration analysis.....	47
3 RESULTS.....	47
3.1 Phylogenetic, coalescence, and migration analyses	47
3.2 Distance analyses	49
4 DISCUSSION	50
5 CONCLUSION	52
ACKNOWLEDGEMENTS.....	52
ORCID	52
R E F E R E N C E S.....	52
SUPPORTING INFORMATION	55
Supporting information	56

CAPÍTULO 2.....	67
Colonization history of <i>Cynopoecilus</i> (Cyprinodontiformes: Rivulidae) across the western margin of the Patos-Mirim Lagoon System, with the description of a new species.....	67
INTRODUCTION	70
MATERIALS AND METHODS	72
Species delimitation and description	73
<i>Morphology-based evaluations</i>	73
<i>Molecular-based evaluations</i>	73
Population structure	74
Evolutionary scenario	74
RESULTS	76
Species delimitation by molecular analyses	76
<i>Phylogenetic analysis</i>	76
<i>Genetic distances</i>	77
Species delimitation by morphological analyses	77
<i>Cynopoecilus</i> sp. n. (Figures 2–4)	77
Holotype	77
Paratype	78
Diagnosis	78
Description	81
<i>External morphology</i>	81
<i>Squamation and contact organs</i>	82
<i>Neuromasts</i>	82
<i>Osteology</i>	82
<i>Colour in life</i>	83
<i>Distribution</i>	83
<i>Etymology</i>	84
<i>Ecological notes</i>	84
<i>Conservation</i>	84
Analyses of population structure	85
Spatio-temporal evolutionary scenario	85
DISCUSSION.....	86
ACKNOWLEDGEMENTS.....	91
REFERENCES.....	92
TABLES.....	97
FIGURES	98
SUPPORTING INFORMATION	107
Supporting Information	108
CAPÍTULO 3.....	119
Hotspots of annual fish along the Patos-Mirim Lagoon System and adjacent areas, with an evaluation of	

conservation priority for species and localities	119
Introduction	122
Materials and methods	125
Studied species or lineages.....	125
Study area	126
Collection and molecular procedures.....	128
Phylogenetic analysis	129
Analysis of endemism, sympatry and richness distribution.....	130
Conservation priority measures.....	130
Results	131
Discussion.....	135
<i>The biological targets of conservation and their priority of conservation</i>	135
<i>Hotspots of diversity of annual fish and their priority of conservation</i>	139
Additional Challenges.....	141
Acknowledgement	143
References.....	143
Figure captions.....	154
Figures	155
Tables	159
CONSIDERAÇÕES FINAIS E PERSPECTIVAS.....	167
REFERÊNCIAS BIBLIOGRÁFICAS	170
ANEXOS	173
Anexo 1 – Publicações extras produzidas pela discente no período do doutorado	173
Anexo 2 – Certificado do Comitê de Ética em Uso Animal	175

LISTA DE FIGURAS

INTRODUÇÃO GERAL

- Figura 1** Mapa de distribuição de *Cynopoecilus*: Laguna dos Patos (*), e localidades tipo de espécies de *C. notabilis* (1), *C. nigrovittatus* (2), *C. intimus* (3), *C. melanotaenia* (4), *C. fulgens* (5), *C. multipapillatus* (6) e *C. feltrini* (7). Modificado de Ferrer; Wingert; Malabarba (2014).....29
- Figura 2** Árvore filogenética de *Austrolebias* gerada por análise bayesiana, baseada em caracteres moleculares (unidade ribossômica 16s, Citocromo b, RAG1, Glyt) e morfológicos. Os valores acima dos ramos são probabilidades posteriores. Fonte: Loureiro et al. (2018). 31

CAPÍTULO 1

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

- 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).....46
- 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.....48
- 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.....49

CAPÍTULO 2

Colonization history of *Cynopoecilus* (Cyprinodontiformes: Rivulidae) across the western margin of the Patos-Mirim Lagoon System, with the description of a new species

- Figure 1** Bayesian Inference tree obtained from the concatenated dataset COI + RAG1 emphasizing the subdivision of *Cynopoecilus* in ten clusters, among which two encompass each of the different populations of *Cynopoecilus* sp. n. Numbers above branches refer to the Bayesian posterior probability of each clade. Colours are in accordance with distribution patterns presented in Figure 5.....98
- Figure 2** Males (A and B) and female (C) paratypes of *Cynopoecilus* sp. n. from Eldorado do Sul municipality, type locality, Rio Grande do Sul, southern Brazil. Photos by Matheus Volcan.....99
- Figure 3** Males paratypes of *Cynopoecilus* sp. n. from Arroio dos Ratos municipality, Rio Grande do Sul, southern Brazil. Photos by Matheus Volcan.....100
- Figure 4** Females paratypes of *Cynopoecilus* sp. n. from Arroio dos Ratos municipality, Rio Grande do Sul, southern Brazil. Photos by Matheus Volcan.....101
- Figure 5** Geographic distribution of *Cynopoecilus* lineages, with an emphasis to the distribution of the two populations of *Cynopoecilus* sp. n. Source: Costa (2002), Costa *et al.* (2016), Ferrer *et al.* (2014) and Garcez *et al.* (2020).....102
- Figure 6** Type locality of *Cynopoecilus* sp. n., a temporary pool in the floodplain of Jacuí River, Eldorado do Sul, Brazil.....103
- Figure 7** Haplotype network constructed with COI sequences of different populations of *C. melanotaenia* and *Cynopoecilus* sp. n. The dashes represent the number of mutational steps. The size of each circle is proportional to haplotype frequencies.....103
- Figure 8** Bayesian chronophylogenetic tree based on COI, ENC1 and RAG1 sequences sampled for two different populations of *Cynopoecilus* sp. n., *C. melanotaenia*, *C. nigrovittatus* and *Cynopoecilus* sp. Values above nodes indicate divergence time estimates. Values below internal branches represent support values, given by posterior probabilities values in Bayesian analysis. Horizontal blue bars represent the 95% HPD values for the estimated dates. This tree was rooted with sequences of *A. wolterstorffi* sampled in both, the east and west margins of the Patos Lagoon, which were also employed to calibrate the molecular clock rates (Garcez *et al.*, 2018).....104
- Figure 9** Spatial diffusion over time showing a north-south colonization route, through different time spans: (A) 150 Kya, (B) 75 Kya, (C) 33 Kya, (D) present. Green: colonization route between *C. nigrovittatus* and *Cynopoecilus* sp. n.; red: colonization route between the two

populations of <i>Cynopoecilus</i> sp. n.; pink: colonization route between <i>Cynopoecilus</i> sp. n. and <i>C. melanotaenia</i> and further dispersion of the later. Figure generated on Google Earth through SPREAD analysis.....	105
Figure 10 Males of representative specimens of <i>Cynopoecilus melanotaenia</i> complex recorded in Brazil (not preserved). (A) <i>C. feltrini</i> ; (B) <i>C. nigrovittatus</i> ; (C) <i>C. melanotaenia</i> ; (D) <i>C. intimus</i> ; (E and F) <i>Cynopoecilus</i> sp. n. Photos by Matheus Volcan.....	106
Figure 11 Females of representative specimens of <i>Cynopoecilus melanotaenia</i> complex recorded in Brazil (not preserved). (A) <i>C. feltrini</i> ; (B) <i>C. nigrovittatus</i> ; (C) <i>C. melanotaenia</i> ; (D) <i>C. intimus</i> ; (E and F) <i>Cynopoecilus</i> sp. n. Photos by Matheus Volcan.....	107

CAPÍTULO 3

Hotspots of annual fish along the Patos-Mirim Lagoon System and adjacent areas, with an evaluation of conservation priority for species and localities

Figure 1 – Map of the Brazilian Rio Grande do Sul state showing the distribution of the 45 municipalities where collections were performed. Some municipalities were sampled in more than one location, totaling 144 collection points or pools. The colors of the symbols represent the different geomorphological units, according to the legend to the right.....	155
Figure 2 – Tree topology generated by Bayesian Analysis for the set of COI and ENC1 sequences characterized for the <i>A. wolterstorffi</i> species complex, the <i>A. adloffii</i> species group and the <i>Cynopoecilus</i> genus, depicting the reciprocal monophyly as well as the phylogenetic positioning of each of the lineages evaluated in this study. Numbers above branches refer to the Bayesian posterior probability of each clade. The dotted lines represent the phylogenetic positioning of lineages recovered with an independent analysis performed with the mitochondrial cytochrome <i>b</i> gene (Volcan et al. 2021).....	156
Figure 3 – Map of the Brazilian Rio Grande do Sul state showing the distribution of the eight strictly endemic lineages. The captions to the right of the figure represent the colors assigned to each species.....	157
Figure 4 – Map of the Brazilian Rio Grande do Sul state showing the distribution of the patterns of lineages richness along the Patos-Mirim Lagoon System. Colors reflect the number of lineages recovered in each grid according to the legend to the right. Numbers are assigned to each grid with three or more registers, to enhance comprehension of text and Table S1.....	158

LISTA DE TABELAS

CAPÍTULO 1

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

TABLE 1	<i>COI</i> 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 <i>Cynopoecilus</i>	50
TABLE 2	<i>RAG1</i> pairwise fixation indices (FST) between lineages of <i>Cynopoecilus</i>	51
Table S1.	List of sampling localities for each of the major lineages recovered in this study, with their respective identification codes and geographical coordinates.....	57

CAPÍTULO 2

Colonization history of *Cynopoecilus* (Cyprinodontiformes: Rivulidae) across the western margin of the Patos-Mirim Lagoon System, with the description of a new species

Table 1	Morphometric data for the holotype and paratypes of <i>Cynopoecilus</i> sp. n.....	97
Table S1	List of sampling localities for specimens used in molecular analysis, with their respective geographical coordinates and GenBank accession numbers.....	109

CAPÍTULO 3

Hotspots of annual fish along the Patos-Mirim Lagoon System and adjacent areas, with an evaluation of conservation priority for species and localities

Table 1	– Summary of the dataset used in the establishment of the studied lineages encompassing the restricted dataset, considering their <i>a priori</i> taxonomic delimitation, the number of sampled localities, the sequences employed for each marker in each analysis and the corresponding <i>a posteriori</i> lineages.....	159
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Table 2 – Summary of the measures of genetic diversity ($Hd*\pi/\mu$), phylogenetic positioning (number of nodes between the root of the tree and the corresponding taxon), and number of populations within and outside Conservation Units (CU) estimated for each lineage of the restricted dataset, with their respective weights ($W_{lineage}$) reflecting conservation priority.....	161
Table 3 – List of temporary pools presenting at least one lineage of the restricted dataset, with their respective weights (W_{pool}) reflecting conservation priority.....	163

INTRODUÇÃO GERAL

A biodiversidade do nosso planeta está diminuindo drasticamente como consequência das ações humanas. A escala do problema é enorme e foi chamada de “sexta extinção em massa” (FRANKHAM; BALLOU; BRISCOE, 2004). A lista vermelha das espécies ameaçadas de extinção divulgada em 2020 pela IUCN, o principal órgão internacional de conservação, indica que mais de 15 mil espécies animais se encontram, atualmente, ameaçadas de extinção, dentre as quais 3.024 (cerca de 20%) constituem espécies de peixes. É consenso, entretanto, que este número representa uma subestimativa, em vista da falta de informações biológicas acerca de muitas espécies e até mesmo da falta de conhecimento taxonômico sobre o real número de espécies atuais (FREELAND; KIRK; PETERSEN, 2011).

A IUCN reconhece a necessidade de conservar a diversidade genética como uma das prioridades globais de conservação. Isso ocorre porque a diversidade genética é a matéria-prima sobre a qual a seleção natural atua para produzir adaptações capazes de lidar com as mudanças ambientais, que constituem um processo contínuo em nosso meio. De fato, a perda de diversidade genética não apenas reduz o potencial evolutivo das populações, como também está associada à redução da aptidão reprodutiva dos indivíduos (FRANKHAM; BALLOU; BRISCOE, 2004). Deste modo, entre as informações relevantes para a compreensão do real *status* de ameaça apresentado por qualquer espécie, merece especial destaque a busca pela compreensão da composição e da organização de sua diversidade genética, que fornece uma medida de seu potencial evolutivo/adaptativo (GALETTI et al., 2009). Essas informações também permitem investigar a evolução das espécies e verificar as afinidades e os limites entre as espécies, o que pode ter consequências importantes na definição das unidades biológicas de conservação e das próprias estratégias de conservação. Afinal, análises de variabilidade genética permitem a comparação e a caracterização de indivíduos, populações e espécies (SOLÉ-CAVA; CUNHA, 2012), em nível isolado ou comparativo.

Nesse sentido, a genética da conservação é um campo que utiliza teorias e técnicas genéticas visando não apenas compreender, mas também diminuir o risco de extinção em espécies ameaçadas (FRANKHAM; BALLOU; BRISCOE, 2004). Tem como objetivo central a utilização de marcadores moleculares e sua interpretação ecológica e evolutiva para auxiliar na diminuição de danos causados às espécies pelas atividades humanas (SOLÉ-CAVA; CUNHA, 2012). Esta é uma área de bastante destaque na biologia da conservação,

apresentando um desenvolvimento crescente ao longo dos últimos anos, e um impacto cada vez maior no estabelecimento de unidades biológicas e/ou geográficas de conservação, bem como de estratégias para a conservação da biodiversidade.

O nível de diversidade genética apresentado por qualquer população ou espécie, bem como sua estruturação, dependem de uma série de fatores, como taxas mutacionais, tamanho efetivo populacional e nível de fluxo gênico (FRANKHAM; BALLOU; BRISCOE, 2004). As populações pequenas sofrem, usualmente, uma forte influência de mecanismos evolutivos do tipo deriva genética, um processo estocástico que leva à redução da diversidade e à fixação de alelos em diferentes loci. Além disso, populações pequenas também costumam apresentar um aumento nas taxas de endocruzamento, o que aumenta os níveis de homozigosidade nas populações (FRANKHAM; BALLOU; BRISCOE, 2004). A reduzida capacidade de dispersão de organismos, ou a presença de barreiras que interfiram na migração, também favorece o isolamento entre as populações, o que reduz o tamanho efetivo da população e aumenta os níveis de endocruzamento (LOUREIRO, 2004). Em conjunto, estas forças reduzem a diversidade genética intrapopulacional, aumentando o risco de colapso mutacional (isto é, o declínio da taxa reprodutiva e espiral descendente em direção à extinção devido à fixação casual de novas mutações ligeiramente deletérias em populações pequenas) e de depressão por endocruzamento (isto é, redução na reprodução, sobrevivência ou outros caracteres quantitativos devido à endogamia) (FRANKHAM; BALLOU; BRISCOE, 2004).

No entanto, em outro nível, estas forças acabam resultando na diferenciação entre populações, que pode culminar em eventos de especiação alopátrica (especiação de populações geograficamente separadas) (RIDLEY, 2006). O grau de diferenciação genética entre as populações também deve influenciar na escolha de estratégias de conservação e manejo de espécies, na qual populações estruturadas geograficamente devem ser utilizadas para definir unidades genéticas prioritárias para conservação (MÉDAIL; BAUMEL, 2018). Em particular, quando as populações estão estruturadas, translocações antrópicas entre estas populações podem não ser desejáveis devido aos riscos de perturbar adaptação locais. A estratégia de conservação deve, pois, procurar preservar a diversidade da espécie em toda a sua distribuição, pois já podem existir adaptações locais que se perderiam no caso de mistura ou extinção dessas populações (FRANKHAM; BALLOU; BRISCOE, 2004). Negligenciar a existência da estrutura filogeográfica dentro das espécies, considerando todas as populações como iguais, levaria ao risco de perda do legado evolutivo insubstituível e do "combustível" para respostas a futuras mudanças ambientais (MÉDAIL; BAUMEL, 2018). Como tal, as análises genéticas de conservação que avaliam os níveis populacionais de variação genética e

níveis de fluxo gênico podem fornecer importantes informações para a gestão de espécies ameaçadas (CAMARGO et al., 2015).

Muitas populações passam, ao longo de sua existência, por períodos de redução significativa do seu tamanho efetivo, através de eventos conhecidos como gargalos de garrafa; outras apresentam tamanhos efetivos reduzidos como uma propriedade demográfica constante (FRANKHAM; BALLOU; BRISCOE, 2004). A filogeografia estuda a distribuição geográfica das linhagens genealógicas ao longo do tempo (AVISE, 2000), buscando avaliar os padrões microevolutivos apresentados pelas populações ao longo do espaço (isto é, caracterizar os níveis e a organização da diversidade) e os processos temporais, históricos ou recorrentes, responsáveis pelos mesmos (BEHEREGARAY, 2008). Um papel da filogeografia é fornecer uma mensagem precisa aos conservacionistas para ajudá-los a incorporar “processos microevolutivos” em suas avaliações a um custo financeiro moderado (DINIZ-FILHO et al., 2008).

Segundo Lévêque et al. (2008), a distribuição atual de peixes foi moldada por milhões de anos, na qual eventos de especiação e extinção foram associados a eventos climáticos e geológicos, proporcionando oportunidades de migração e colonização de novos habitats por diferentes espécies. A mutação, a seleção natural, a deriva genética e o fluxo gênico constituem os processos evolutivos recorrentes responsáveis pelos padrões microevolutivos (RIDLEY, 2006). Alterações ambientais associadas a eventos paleogeográficos ou paleoclimáticos constituem eventos históricos que podem interferir na atuação dos processos recorrentes. A inferência dos processos microevolutivos responsáveis pelo estabelecimento dos padrões filogeográficos no espaço e no tempo permite avaliar a influência de diferentes eventos na demografia das espécies, o que pode ser útil não apenas na caracterização do passado, mas também na compreensão do presente e até na gestão do futuro.

No entanto, muitas vezes, é também importante buscar compreender os padrões e processos macroevolutivos, associados ao estudo da evolução a partir da escala de conjuntos de genes independentes, onde o foco são as mudanças que ocorrem no nível de espécie ou acima (RIDLEY, 2006). Neste âmbito, a informação genética auxilia na resolução de incertezas taxonômicas e na definição de unidades de gestão dentro de espécies, visto que a biodiversidade pode estar escondida na forma de espécies crípticas, que inicialmente parecem morfologicamente indistinguíveis, mas são compostas de duas ou mais espécies distintas (FRANKHAM; BALLOU; BRISCOE, 2004). De fato, muitas vezes a biologia molecular revela a existência de espécies crípticas dentro do que os taxônomos haviam considerado uma única espécie, o que gera consequências importantes para a conservação (BICKFORD et al.,

2007). Além das limitações da taxonomia morfológica clássica, associada à existência de espécies crípticas, o chamado impedimento taxonômico, ocasionado pela escassez de taxônomos e a deficiência de financiamentos estão associadas a uma importante subestimativa da biodiversidade (SOLÉ-CAVA; CUNHA, 2012).

Também é importante considerar que, muitas vezes, os processos macroevolutivos refletem apenas uma extensão dos processos microevolutivos (RIDLEY, 2006), de modo que a compreensão dos fenômenos a longo prazo requer uma interação entre as duas ênfases. Por fim, em nível macroevolutivo, a análise das relações filogenéticas permite não apenas revisar questões taxonômicas, como também avaliar os cenários espaço-temporais associados a diferentes eventos de diversificação/extinção dentro de um grupo de espécies. Existem, por exemplo, diferentes propostas que visam mensurar a prioridade de conservação de espécies com base no seu posicionamento filogenético (p. ex. VANE-WRIGHT et al., 1991) e de habitats com base em sua diversidade filogenética (CADOTTE; DAVIES, 2010). Quando associados, estudos de nível macro e microevolutivo podem ainda permitir o estabelecimento de padrões concordantes, ocasionados por processos similares que afetaram diferentes espécies em uma mesma região. Isso pode fornecer informações úteis para a compreensão da origem da biodiversidade local, e para o estabelecimento de unidades geográficas de conservação.

Áreas úmidas e peixes anuais

Tendo em vista que as áreas úmidas são áreas prioritárias para a conservação da biodiversidade, conhecer e conservar a diversidade genética de suas espécies é de fundamental importância. Peixes anuais certamente figuram entre as espécies de vertebrados mais ameaçadas de extinção, especialmente em regiões com maior abundância de áreas úmidas (VOLCAN; LANÉS, 2018). No Rio Grande do Sul, por exemplo, cerca de 60% das espécies de peixes de água doce ameaçadas de extinção é composta por peixes anuais (RIO GRANDE DO SUL, 2014). A peculiar distribuição de espécies de peixes anuais, que constituem populações pequenas e isoladas, instaura um cenário propício para a forte atuação de mecanismos do tipo deriva genética. Este fator, acrescido à grande dificuldade de fluxo gênico entre as populações, tende a elevar os níveis de endogamia e de estruturação genética, aumentando ainda mais a vulnerabilidade intrínseca destas populações.

Além disso, a perda extensa de habitats e a fragmentação das áreas úmidas de água doce representam uma ameaça para diferentes componentes da ictiofauna, em especial para

peixes anuais. Isto ocorre porque entre os tipos de áreas úmidas, as áreas úmidas temporárias (habitadas por estes peixes) são especialmente vulneráveis, devido ao tamanho reduzido, pouca profundidade e ao fato de encontrarem-se secas durante parte do ano (DUDLEY, 2006). De fato, a extensão dos charcos que suportam a diversidade de peixes anuais neotropicais vem sendo amplamente reduzida, em virtude da expansão de atividades agrícolas ou urbanas, ou mesmo devido à construção e duplicação de estradas (FONTANA; BENCKE; REIS, 2003; LANÉS; MALTCHIK, 2010; VOLCAN; LANÉS; GONÇALVES, 2009; VOLCAN; LANÉS; CHEFFE, 2010). Diante deste conjunto de ameaças, foi criado o “Plano de Ação Nacional para a Conservação dos Peixes Rivulídeos Ameaçados de Extinção - PAN Rivulídeos” no Brasil (ICMBio, 2013). Neste documento, a importância da realização de estudos técnicos e científicos, *in situ* e *ex situ*, aplicados à conservação das espécies focais de rivulídeos e seus habitats foi enfatizada. Afinal, estes peixes são considerados importantes componentes para o equilíbrio das áreas úmidas, pois são os predadores de topo dos charcos temporários, com hábito alimentar oportunista, se alimentando principalmente de invertebrados (LAUFER et al., 2009; GONÇALVES; SOUZA; VOLCAN, 2011)

Rivulidae está entre as famílias mais diversificadas de peixes Neotropicais de água doce, com cerca de 468 espécies descritas (FRICKE; ESCHMEYER; VAN DER LAAN, 2021). Representantes desta família estão distribuídos na América do Sul, Central e sul dos Estados Unidos, com cerca de um terço das espécies ocorrendo em território brasileiro (COSTA, 2008; VOLCAN et al., 2015). A maior parte dos membros desta família são popularmente conhecidos como peixes anuais ou *killifish* sazonais, pois completam o seu ciclo de vida em áreas alagáveis temporárias que secam sazonalmente, apresentando adaptações que propiciam sua sobrevivência nestes ambientes extremos (MYERS, 1952). Isto ocorre porque seus ovos são resistentes à dessecação, permanecendo enterrados no substrato, em estágio de diapausa, até o início do alagamento do charco, quando iniciam um novo ciclo de vida (WOURMS, 1972). Além disso, o apetite voraz, a maturação sexual precoce, a alta fecundidade e o curto ciclo de vida são características típicas de peixes anuais (FURNESS, 2015) e evidenciam sua complexa adaptação aos ambientes efêmeros onde vivem.

Entre os rivulídeos encontrados na região sul do Brasil, destacam-se aqueles do gênero *Cynopoecilus* Regan, 1912 por apresentar fertilização interna. Esta ocorre com o casal nadando lentamente no meio da coluna d’água, lado a lado, quando o macho envolve as fêmeas com as nadadeiras dorsal e anal, transferindo os espermatozoides diretamente para o trato urogenital feminino por meio de estruturas especializadas presentes na nadadeira anal masculina (chamadas de papila urogenital). A desova ocorre mais tarde, com a fêmea sozinha

depositando seus ovos no substrato (COSTA; AMORIM; MATTOS, 2016a). Este gênero compreende, atualmente, seis espécies, sendo cinco endêmicas do Sistema Lagunar Patos-Mirim (SLPM) e áreas costeiras adjacentes, ocorrendo em charcos próximos de corpo d'água lóticos do sul do Brasil ao leste do Uruguai e uma endêmica da bacia do baixo rio Tubarão, em Santa Catarina, sul do Brasil (Figura 1) (COSTA; AMORIM; MATTOS, 2016b; FERRER; WINGERT; MALABARBA, 2014). São elas: *C. melanotaenia*, a espécie tipo do gênero, *C. fulgens*, *C. intimus*, *C. nigrovittatus*, *C. notabilis* e *C. feltrini*, respectivamente (COSTA, 2002; COSTA; AMORIM; MATTOS, 2016b; FERRER; WINGERT; MALABARBA, 2014). Até recentemente, eram consideradas sete espécies dentro do gênero; no entanto, Costa (2016), em uma revisão taxonômica do gênero, indicou que *C. multipapillatus* é sinonímia de *C. fulgens*. Quanto ao *status* de ameaça, *C. intimus* e *C. fulgens* são consideradas vulneráveis de extinção (VU) pela Lista de Espécies da Fauna Silvestre Ameaçadas de Extinção no Estado do Rio Grande do Sul (RIO GRANDE DO SUL, 2014) e pelo livro vermelho da fauna ameaçada de extinção do Brasil (ICMBio, 2018), e também segundo a lista vigente das Espécies da Fauna Brasileira Ameaçadas de Extinção (BRASIL, 2014). Por outro lado, *C. nigrovittatus* encontra-se na categoria VU apenas na Lista do Estado do Rio Grande do Sul (RIO GRANDE DO SUL, 2014). Já *C. notabilis* não está incluída na lista vermelha por ocorrer dentro de uma unidade de conservação (FERRER; WINGERT; MALABARBA, 2014).

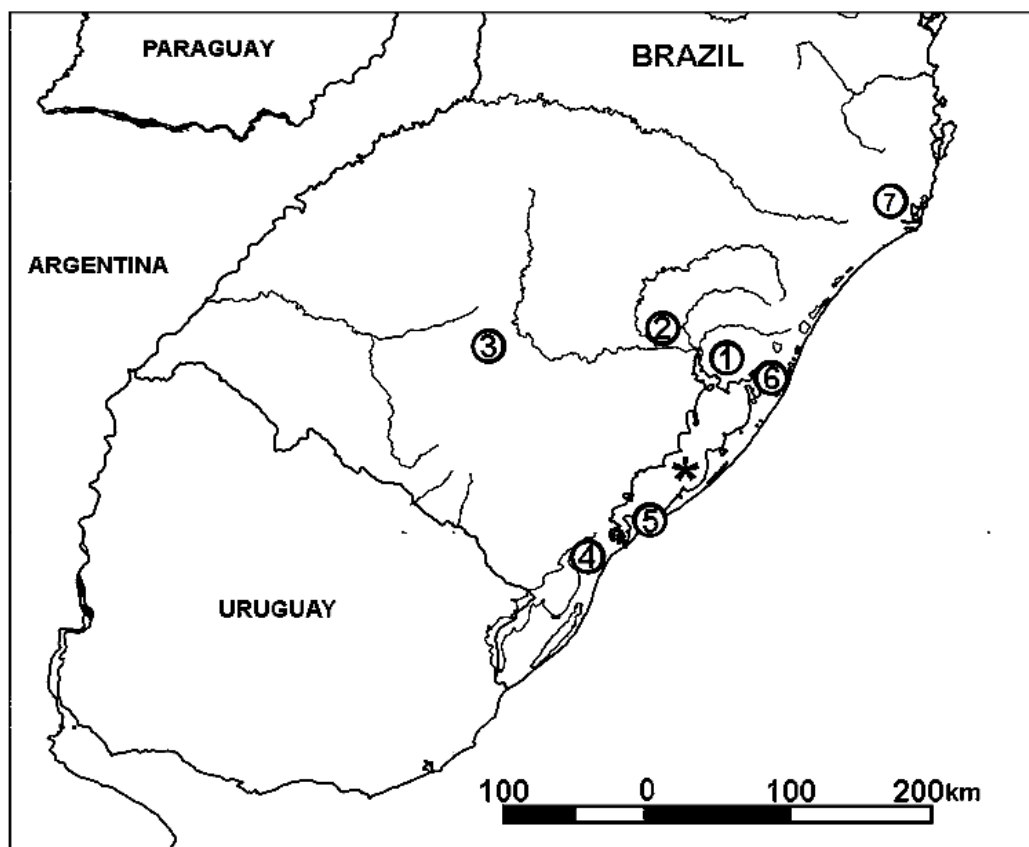


Figura 1 Mapa de distribuição de *Cynopoecilus*: Laguna dos Patos (*), e localidades tipo de espécies de *C. notabilis* (1), *C. nigrovittatus* (2), *C. intimus* (3), *C. melanotaenia* (4), *C. fulgens* (5), *C. multipapillatus* (6) e *C. feltrini* (7). Modificado de Ferrer; Wingert; Malabarba (2014).

Comparado a *Cynopoecilus*, *Austrolebias* Costa, 1998 constitui um gênero mais especioso, diverso e amplamente distribuído. Este gênero compreende cerca de 50 espécies de peixes anuais (LANÉS; VOLCAN; MALTCHIK, 2021; VOLCAN et al., 2021), distribuídas nas bacias La Plata e Patos-Mirim (LOUREIRO; DE SÁ, 2015), e também na bacia Amazônica (NIELSEN; PILLET, 2015). A monofilia de *Austrolebias* vem sendo suportada tanto por análises morfológicas (COSTA, 2006), quanto por estudos moleculares (GARCÍA et al., 2000; GARCÍA et al., 2014; LOUREIRO et al., 2018). Segundo Costa (2006), *Austrolebias* apresenta três espécies basais (*A. luteoflammulatus*, *A. gymnoventris* e *A. jaegari*) e cinco grupos de espécies (*A. robustus*, *A. elongatus*, *A. alexandri*, *A. bellottii* e *A. adloffii*). No entanto, hipóteses mais recentes têm posicionado *A. luteoflammulatus* junto com *A. quirogai* como um clado derivado dentro do gênero (GARCÍA et al., 2014; LOUREIRO et al., 2018). Costa (2006) propôs a elevação de cada um destes grupos ao status de subgênero e Loureiro et al. (2018) utilizou essa nomenclatura dos subgêneros (Figura 2).

Dentro de *Austrolebias*, o grupo de espécies *Austrolebias adloffi*, ocorre majoritariamente ao longo do SLPM, em territórios brasileiros e uruguaios (COSTA, 2006; GARCÍA et al., 2009; GARCÍA; RÍOS; GUTIÉRREZ, 2015). Dentro do território brasileiro e ao longo do SLPM observa-se a ocorrência de sete espécies deste grupo: *A. adloffi*, *A. arachan*, *A. bagual*, *A. charrua*, *A. minuano*, *A. nachtigalli* e *A. nigrofasciatus* (VOLCAN et al., 2015). Destas espécies, pelo menos três (*A. charrua*, *A. minuano* e *A. nigrofasciatus*) aparecem como “em perigo” (EN) e quatro (*A. arachan*, e *A. bagual*) como “criticamente em perigo” (CR) junto ao Livro Vermelho da Fauna Brasileira Ameaçada de Extinção e na Lista de Espécies da Fauna Silvestre Ameaçadas de Extinção no Estado do Rio Grande do Sul (ICMBio, 2018; RIO GRANDE DO SUL, 2014; VOLCAN; LANÉS; GONÇALVES, 2014). Além disso, *A. adloffi* e *A. nachtigalli* aparecem na categoria EN no Livro Vermelho nacional (ICMBio, 2018) e na categoria CR na Lista de Espécies do Estado (RIO GRANDE DO SUL, 2014).

O grupo de *Austrolebias elongatus* (subgênero *Megalebias*), por outro lado, compreende cinco espécies: *A. cheradophilus*, *A. wolterstorffi*, *A. prognathus*, *A. monstrosos* e *A. elongatus*. Destas, *A. wolterstorffi* constitui uma das espécies com maior amplitude de distribuição dentro do gênero, ocorrendo a partir do norte da Laguna dos Patos até o sul da Lagoa Mirim (LOUREIRO; DE SÁ, 2015). Ainda assim, é considerada Criticamente Em Perigo (CR) de extinção pelos livros vermelhos da fauna ameaçada de extinção do Rio Grande do Sul (RIO GRANDE DO SUL, 2014) e do Brasil (ICMBio, 2018). Este status é ainda agravado pelo fato de que esta espécie se mostrou subdividida em seis grupos de populações, que constituem duas grandes linhagens com distribuição restrita à margem leste ou oeste da Laguna dos Patos (GARCEZ et al., 2018). Além de *A. wolterstorffi*, duas outras espécies do grupo *elongatus* são frequentemente amostradas ao longo do SLPM: *A. cheradophilus* e *A. prognathus*. Ambas constam como CR nos livros vermelhos da fauna ameaçada de extinção (ICMBio, 2018; RIO GRANDE DO SUL, 2014).

Outras espécies de *Austrolebias* que ocorrem no SLPM e áreas adjacentes, no estado do Rio Grande do Sul, são *A. camaquensis*, *A. cyaneus*, *A. juanlangi*, *A. litzi* e *A. paucisquama* (grupo de *A. alexandri*, subgênero sem nome), *A. jaegari* e *A. aff. gymnoventris* (grupo *A. jaegari* + *A. gymnoventris*, subgênero *Gymnolebias*), *A. luteoflammulatus* e *A. quirogai* (grupo *A. luteoflammulatus* + *A. quirogai*, subgênero *Acantholebias*) e *A. melanoorus* e *A. univentripinnis* (grupo *A. bellottii*, subgênero *Austrolebias*). Destas espécies, uma (*A. melanoorus*) aparece nas listagens como “em perigo” (EN) e oito (*A. cyaneus*, *A. jaegari*, *A. juanlangi*, *A. litzi*, *A. luteoflammulatus*, *A. paucisquama*, *A. quirogai* e *A.*

LOUREIRO; TASSINO, 2008). O comportamento mais frequentemente observado nos machos é a “exibição lateral”, na qual características morfológicas e de colorido são exibidas para a fêmea. Além dos sinais visuais, os machos vibram as nadadeiras dorsal e anal e ondulam o corpo. Esses movimentos de cortejo são altamente conservados dentro de *Austrolebias* (GARCÍA; LOUREIRO; TASSINO, 2008).

Área de estudo

O SLPM, localizado no sul do Brasil, é uma das áreas com maior número de espécies endêmicas de peixes anuais da região subtropical do Neotrópico (COSTA, 2002), abrigando pelo menos 33 espécies de peixes anuais (GARCEZ et al., 2020; LANÉS; VOLCAN; MALTCHICK, 2021; VOLCAN et al., 2015, 2021). Muitas espécies de peixes anuais são amplamente influenciadas pela presença dos diferentes corpos d’água presentes nesta região, com destaque para a Laguna dos Patos e a Lagoa Mirim. Este é o caso, por exemplo, das diferentes linhagens de *A. minuano/charrua* (FERNANDES et al., 2021) de *A. wolterstorffi* (GARCEZ et al., 2018). Neste último caso, os padrões espaço-temporais de diversificação são congruentes com os eventos paleogeográficos que afetaram a Planície Costeira do Rio Grande do Sul durante o Pleistoceno. Por outro lado, para *A. minuano/charrua*, é possível que eventos de migração e diferenciação tenham sido influenciados pela presença de paleocanais que cruzaram a região antes da formação da Laguna dos Patos (WESCHENFELDER et al., 2010).

Historicamente, essa região foi bastante impactada pelas transgressões e regressões marinhas do Pleistoceno e pós-Pleistoceno, resultando em modificações e fragmentações (SPRECHMAN, 1978). Durante todo este período, a Planície Costeira do Rio Grande do Sul foi retrabalhada, na forma de quatro grandes sistemas deposicionais Laguna-Barreira (TOMAZELLI; VILLWOCK, 2005): o primeiro Sistema Depositional ocorreu há cerca de 400 mil anos atrás, e ocupa os terrenos mais antigos, na região da Bacia do Paraná e de Porto Alegre; o segundo Sistema ocorreu há aproximadamente 325 mil anos e foi responsável pela implantação inicial da Laguna dos Patos e pelo isolamento da Lagoa Mirim; o terceiro Sistema ocorreu há cerca de 120 mil anos, e resultou na implantação final da Laguna dos Patos; o quarto Sistema decorre da última transgressão pós-glacial, e data de somente cinco mil anos atrás (GARCÍA-RODRIGUEZ; WITKOWAKI, 2003; TOMAZELLI & VILLWOCK, 2005).

Segundo Loureiro (2004), como a última destas transgressões é muito recente, muitas das espécies de peixes anuais dessa costa, assim como sua variabilidade intraespecífica,

podem ter origem relativamente recente, relacionadas a estes eventos geológicos. Isto vem sendo suportado, em diferentes níveis para diferentes espécies (FERNANDES et al., 2021; GARCEZ et al., 2018). Estes estudos demonstram que questões sobre a origem da grande diversidade Neotropical só podem ser abordadas à luz das relações evolutivas entre as diferentes unidades taxonômicas ou populacionais. De fato, eventos de divergência entre as espécies de peixes anuais que ocorrem ao longo do SLPM podem ser estimados utilizando os eventos de formação da Planície Costeira como calibrações para relógios moleculares, possibilitando assim, a avaliação do cenário espaço-temporal associado a evolução destas e de outras espécies.

Ferramentas de estudo

As características intrínsecas dos peixes anuais, aliadas às características estocásticas do ambiente em que vivem e aos mecanismos evolutivos são capazes de gerar uma alta variabilidade morfológica e molecular. Portanto, a utilização de marcadores moleculares e morfológicos pode auxiliar na delimitação de espécies, na caracterização da estruturação genética de populações, e no estabelecimento das relações filogenéticas entre as linhagens, auxiliando no estabelecimento de estratégias de conservação das espécies.

Taxonomia integrativa: combinando dados morfológicos e moleculares

Como uma das primeiras etapas em qualquer estudo biológico é a avaliação e identificação das espécies existentes, os estudos taxonômicos são imprescindíveis também para a biologia da conservação. O DNA barcode veio auxiliar nesta tarefa, ao propor o uso de uma pequena sequência do gene citocromo c oxidase 1 (COI) para a identificação de espécies previamente descritas e para a descoberta e diagnóstico de novas espécies. Este gene foi escolhido por representar um marcador molecular espécie-específico, que apresenta grande variabilidade entre diferentes espécies (HEBERT et al., 2003). O DNA barcode vem desempenhando um papel importante na descoberta e proposição de espécies novas em diferentes grupos, entre os quais, em peixes anuais (COSTA; AMORIM, 2011; COSTA; AMORIM; ARANHA, 2014; COSTA; AMORIM; MATTOS, 2016b), de forma que o mesmo apresenta uma aplicação bastante voltada para a biologia da conservação. Afinal, a existência de diversidade genética escondida na forma espécies crípticas pode causar vários vieses regionais e taxonômicos nas estimativas de diversidade, dificultando a criação de estratégias de conservação das espécies (BICKFORD et al., 2007; WIENS, 2007).

É importante enfatizar que o DNA barcode apenas sugere a existência de novas espécies, sendo visto como um suporte para a taxonomia e não como uma prova definitiva. Desta forma, o mesmo deve ser usado em conjunto com outros marcadores, que tornam a delimitação das espécies mais confiável (DE SALLE, 2006; WIEMERS; FIEDLER 2007). Algumas vezes, o uso de outros marcadores moleculares, especialmente aqueles com evolução independente, associados a genes nucleares, pode fornecer maior suporte às hipóteses inicialmente fornecidas por COI. No entanto, a adição de dados não genéticos, como os ecológicos ou morfológicos, é essencial para propor hipóteses robustas envolvendo a presença e a descrição de espécies novas (PUILLANDRE et al., 2012). De fato, o DNA barcode depende da taxonomia tradicional para inferências sobre a sistemática de qualquer táxon e para a aceitação dos resultados no corpo de conhecimento científico (KVIST, 2013).

Hotspots de biodiversidade

Os *hotspots* de biodiversidade constituem uma ferramenta útil para definir prioridades de conservação, uma vez que desempenham um papel importante na tomada de decisões com um menor custo para preservar a biodiversidade (MARCHESE, 2015; MYERS et al., 2000). O conceito de *hotspot* está intimamente ligado ao conceito de biodiversidade e é usado frequentemente na literatura de biologia e conservação com significados diferentes. Enquanto no sentido estrito, o significado é baseado em uma estimativa de espécies endêmicas e de perda de habitat, em um sentido amplo, refere-se a qualquer área ou região com biodiversidade excepcionalmente alta em nível de ecossistema, espécie e genética (MARCHESE, 2015).

Essas áreas são geralmente definidas por uma ou mais métricas baseadas em espécies (riqueza de espécies, riqueza de espécies endêmicas e número de espécies ameaçadas) ou com foco na diversidade filogenética e funcional, a fim de proteger as espécies que suportam papéis únicos e insubstituíveis dentro do ecossistema. Em particular, o planejamento de conservação tem usado informações de riqueza de espécies combinadas com diferentes medidas de insubstituibilidade (por exemplo, endemismo) para priorizar algumas regiões. Frequentemente, no entanto, essas análises têm omitido peixes devido à falta de dados confiáveis (MARCHESE, 2015).

A diversidade filogenética vem mostrando-se uma abordagem mais integrativa que quantifica as informações evolutivas representadas em termos de grupos de táxons. Recentemente, D'Agata et al. (2014) mostraram que, apesar de uma perda mínima de riqueza

de espécies de peixes que pode ocorrer ao longo de um gradiente de pressão humana, muitas funções e linhagens filogenéticas podem ser perdidas. Portanto, a diversidade filogenética pode ser mais útil do que a riqueza de espécies na manutenção dos serviços ecossistêmicos (CADOTTE; DAVIES, 2010), fornecendo informações adicionais para orientar a tomada de decisão de conservação.

Outra informação importante neste âmbito pode ser fornecida pela análise do posicionamento filogenético das espécies. Neste caso, a prioridade de conservação é inferida a partir da topologia de uma árvore filogenética, favorecendo táxons basais não pertencentes a linhagens específicas (VANE-WRIGHT et al., 1991). No Rio de Janeiro, em um estudo com cinco espécies de killifishes, Costa (2012) avaliou os níveis de prioridade de conservação para cada área com base no posicionamento filogenético de suas espécies, seguindo o método inicialmente descrito por Vane-Wright et al. (1991).

Assim, fica evidente a importância da demarcação de *hotspots*, que busquem delimitar locais com maiores níveis de endemismo, simpatria e riqueza, avaliando as prioridades de conservação das linhagens genéticas e suas áreas de ocorrência. Esse mapeamento pode, pois, fornecer subsídios para a formulação e implementação de políticas públicas voltadas ao estabelecimento de áreas de conservação *in situ* de espécies e suas comunidades associadas.

OBJETIVO GERAL

Auxiliar na delimitação de estratégias de conservação *in situ* de peixes anuais dos gêneros *Cynopoecilus* e *Austrolebias*, através do esclarecimento de questões taxonômicas, micro e macroevolutivas.

Objetivos específicos

- 1 – Avaliar a presença de monofilia recíproca em cada uma das seis espécies de *Cynopoecilus*, validando seu status taxonômico;
- 2 – Avaliar a presença de diversidade críptica dentro do gênero *Cynopoecilus* e descrever novas espécies, quando necessário;
- 3 – Analisar as relações filogenéticas e reconstruir o cenário espaço-temporal associado à invasão/colonização entre espécies de *Cynopoecilus* no SLPM;
- 4 – Inferir *hotspots* para a conservação de *Cynopoecilus* e *Austrolebias* no SLPM, com base no estabelecimento do menor número de pontos capazes de sustentar a maior variabilidade para o conjunto dos grupos taxonômicos/evolutivos considerados;

5 – Comparar os padrões de distribuição da diversidade genética em *C. melanotaenia* e pelo menos mais uma espécie proximamente relacionada.

HIPÓTESES

- 1 – As seis espécies descritas de *Cynopoecilus* são válidas e evolutivamente independentes;
- 2 – Existem sinais de estruturação condizentes com a presença de diversidade críptica, especialmente em *C. fulgens*, que apresenta uma ampla distribuição em charcos ao longo da borda externa da Laguna dos Patos e em *C. nigrovittatus*, que apresenta populações isoladas pelo Rio Jacuí. Neste caso, dada a baixa capacidade de dispersão desses peixes, a variabilidade molecular e morfológica está correlacionada e estruturada geograficamente;
- 3 – As relações filogenéticas e a invasão/colonização/diversificação dentro de *Cynopoecilus* refletem a ordem de formação dos quatro grandes sistemas deposicionais Laguna-Barreira na Planície Costeira, com sinais de vicariância associados a atuação da Laguna dos Patos e do Rio Jacuí como barreiras ao fluxo gênico;
- 4 – A diversidade de peixes anuais dos gêneros *Cynopoecilus* e *Austrolebias* encontra-se distribuída de forma heterogênea ao longo do SLPM, permitindo o estabelecimento de *hotspots* de biodiversidade;
- 5 – Diferentes espécies de *Cynopoecilus* apresentam padrões semelhantes de distribuição da diversidade genética ao longo do SLPM, com baixos níveis de diversidade intrapopulacional, devido ao tamanho populacional reduzido e a ausência de fluxo gênico entre populações, e altos níveis de diferenciação interpopulacional, devido à ausência de fluxo gênico entre populações.

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CAPÍTULO 1

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

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

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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;

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Machado et al., 2017; Witt, Threlhoff, & 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 & Cicero, 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 Manguera 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, Poláček, & 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

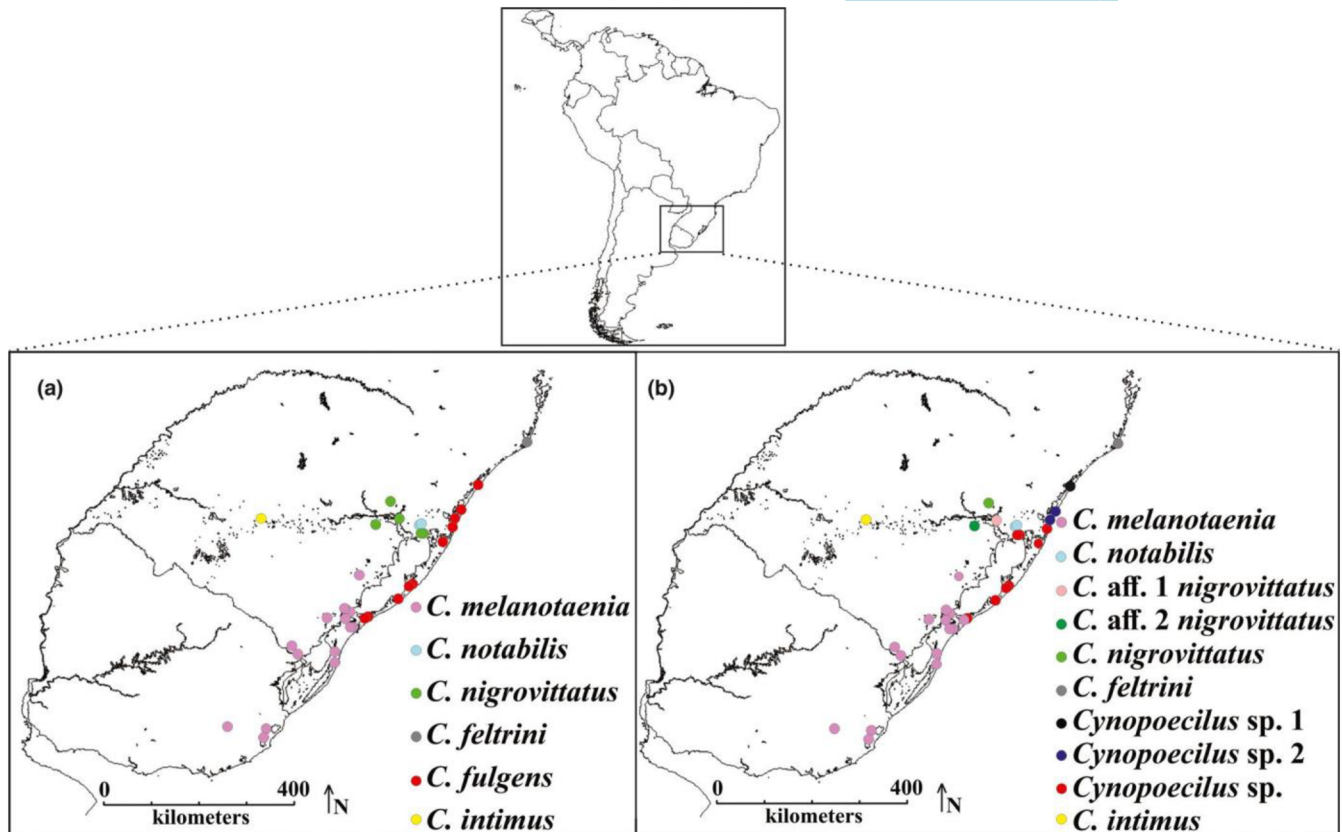


FIGURE 1 Distribution map of the 34 sampling sites of *Cynopoecilus*, emphasizing differences between traditional species boundaries (a) and lineages defined after this study (b)

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, Fritsch, & 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 MgCl₂, 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 (C₂H₇NO₂) 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 ucl.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://www.abi.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)

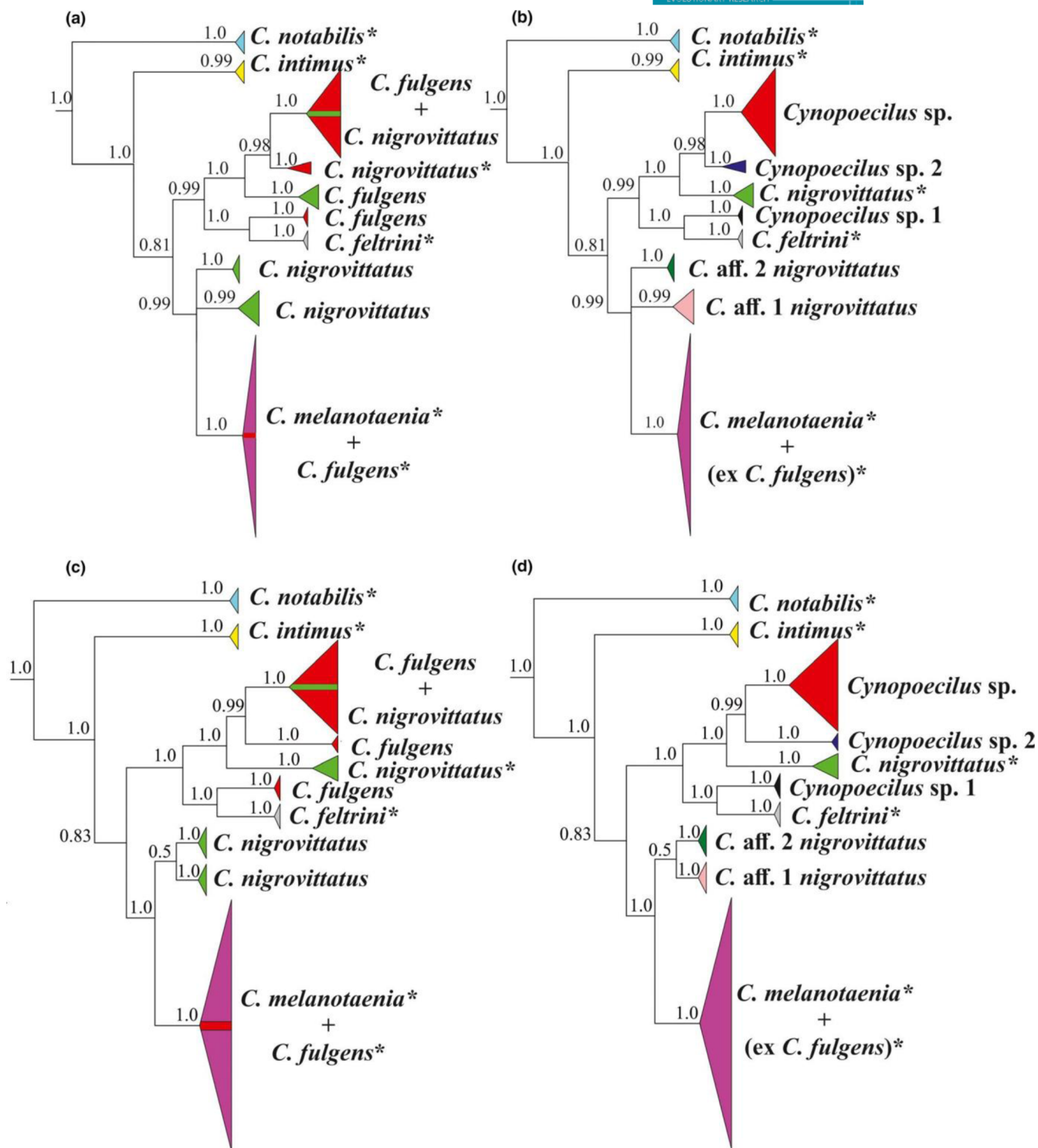


FIGURE 2 Tree topology generated by Bayesian Inference of *COI* (a, b) and *COI* + *RAG1* (c, d) sequences of *Cynopoecilus* emphasizing differences between traditional species boundaries (a, c) and lineages defined after this study (b, d). Numbers above branches refer to the Bayesian posterior probability 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 Lamarck. 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 Lamarck are given as a function of $M = m/\mu$, where m is the chance for a lineage to migrate per generation, and μ 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 F_{ST} 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

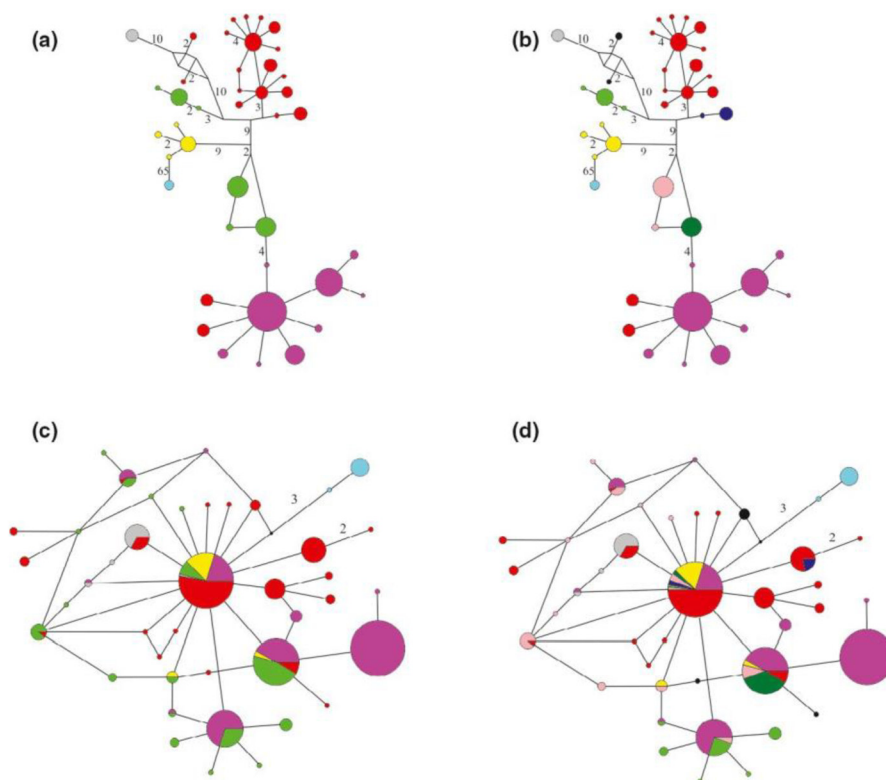


FIGURE 3 Haplotype network constructed with *COI* (a, b) and *RAG1* (c, d) sequences of *Cynopoecilus* emphasizing differences between traditional species boundaries (a, c) and lineages defined after this study (b, d). Number of step-mutations higher than one is in brackets. The size of each circle is 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 <i>C. melanotaenia</i>	0.002	0.948*	0.889*	0.857*	0.959*	0.987*	0.949*	0.957*	0.951*	0.937*
2 <i>C. intimus</i>	0.040	0.001	0.966*	0.971*	0.970*	0.990*	0.973*	0.952*	0.971*	0.922*
3 <i>C. aff. 1 nigrovittatus</i>	0.021	0.030	0.002	0.948*	0.985*	0.998*	0.990*	0.970*	0.987*	0.910*
4 <i>C. aff. 2 nigrovittatus</i>	0.021	0.030	0.012	0	0.990*	1.000*	1.000*	0.976*	0.995*	0.908*
5 <i>C. nigrovittatus</i>	0.058	0.056	0.056	0.057	0.001	0.995*	0.981*	0.947*	0.960*	0.830*
6 <i>C. notabilis</i>	0.202	0.198	0.196	0.197	0.205	0	1.000*	0.977*	0.998*	0.975*
7 <i>C. feltrini</i>	0.060	0.056	0.052	0.051	0.055	0.210	0	0.911*	0.991*	0.897*
8 <i>Cynopoecilus</i> sp. 1	0.067	0.064	0.054	0.055	0.051	0.207	0.037	0.009	0.936*	0.890*
9 <i>Cynopoecilus</i> sp. 2	0.053	0.057	0.049	0.050	0.031	0.213	0.049	0.047	0.002	0.722*
10 <i>Cynopoecilus</i> 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	<i>C. melanotaenia</i>	0.280*	0.194*	0.153*	0.347*	0.702*	0.365*	0.264*	0.379*	0.275*
2	<i>C. intimus</i>		0.122*	0.492*	0.520*	0.834*	0.538*	0.418*	0.507*	0.369*
3	<i>C. aff. 1 nigrovittatus</i>			0.295*	0.341*	0.684*	0.340*	0.160*	0.292*	0.230*
4	<i>C. aff. 2 nigrovittatus</i>				0.671*	0.882*	0.706*	0.436*	0.644*	0.390*
5	<i>C. nigrovittatus</i>					0.909*	0.671*	0.606*	0.675*	0.494*
6	<i>C. notabilis</i>						0.955*	0.834*	0.879*	0.709*
7	<i>C. feltrini</i>							0.673*	0.744*	0.451*
8	<i>Cynopoecilus</i> sp. 1								0.310*	0.207*
9	<i>Cynopoecilus</i> sp. 2									0.158*
10	<i>Cynopoecilus</i> 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 suggest 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 tracts (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).

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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.

[Correction added on 10 July 2020, after Online publication: Surname of all the authors have been corrected in this current version.]

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Supporting information

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

Daiana Kaster Garcez, Murilo de Oliveira Fernandes, Gabrielli da Rosa Ozório, Matheus Vieira Volcan and Lizandra Jaqueline Robe

Alignment S1. *COI* alignment.

Alignment S2. *RAG1* alignment.

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

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

N	Identification code	Initial ID	New ID	Population	Coordinates	GenBank (COI)	GenBank (RAG1)	Voucher
1	16RU1	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Uruguay Ruta 16	-34.031301 -53.823860	MK836470	MK836730	
2	16RU2	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Uruguay Ruta 16	-34.031301 -53.823860	MK836471	MK836731	
3	16RU3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Uruguay Ruta 16	-34.031301 -53.823860	MK836472	MK836732	
4	16RU4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Uruguay Ruta 16	-34.031301 -53.823860	MK836473	MK836733	
5	RO1	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Uruguay Laguna Negra	-34.005554 -53.585891	MK836677	MK836937	
6	RO3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Uruguay Laguna Negra	-34.005554 -53.585891	MK836678	MK836938	
7	RO4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Uruguay Laguna Negra	-34.005554 -53.585891	MK836679	MK836939	
8	RO5	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Uruguay Laguna Negra	-34.005554 -53.585891	MK836680	MK836940	
9	RO6	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Uruguay Laguna Negra	-34.005554 -53.585891	MK836681	MK836941	
10	RO7	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Uruguay Laguna Negra	-34.005554 -53.585891	MK836682	MK836942	
11	RU9	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Uruguay Ruta 9	-34.213551 -53.883296	MK836683	MK836943	
12	10T1	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM10	-32.554250 -52.495500	MK836466	MK836726	MCP** 54235
13	10T2	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM10	-32.554250 -52.495500	MK836467	MK836727	MCP 54235
14	10T3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM10	-32.554250 -52.495500	MK836468	MK836728	MCP 54235
15	10T4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM10	-32.554250 -52.495500	MK836469	MK836729	MCP 54235
16	22T1	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836474	MK836734	MCP 54235
17	22T10	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836475	MK836735	MCP 54235
18	22T2	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836476	MK836736	MCP 54235
19	22T3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836477	MK836737	MCP 54235
20	22T4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MN326791	MK836738	MCP 54235
21	22T5	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836478	MK836739	
22	22T6	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836479	MK836740	
23	22T7	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836480	MK836741	
24	22T8	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836481	MK836742	
25	22T9	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836482		
26	CP1	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Capão do Leão	-31.892497 -52.656845	MK836510	MK836770	MCP 54236

27	CP2	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Capão do Leão	-31.892497 -52.656845	MK836511	MK836771	MCP 54236
28	CP3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Capão do Leão	-31.892497 -52.656845	MK836512	MK836772	MCP 54236
29	CP4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Capão do Leão	-31.892497 -52.656845	MK836513	MK836773	MCP 54236
30	CP5	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Capão do Leão	-31.892497 -52.656845	MK836514	MK836774	
31	CP6	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Capão do Leão	-31.892497 -52.656845		MK836996	
32	F1	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836515	MK836775	
33	F10	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836516	MK836776	
34	F11	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836517	MK836777	
35	F12	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836518	MK836778	MCP 54237
36	F13	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836519	MK836779	MCP 54237
37	F14	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836520	MK836780	MCP 54237
38	F15	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836521	MK836781	MCP 54237
39	F16	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836522	MK836782	
40	F17	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836523	MK836783	
41	F18	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836524	MK836784	
42	F19	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836525	MK836785	
43	F2	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836526	MK836786	
44	F20	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836527	MK836787	
45	F21	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836528	MK836788	
46	F3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836529	MK836789	
47	F4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836530	MK836790	
48	F5	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836531	MK836791	
49	F6	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836532	MK836792	
50	F8	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836533	MK836793	
51	R1	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836660	MK836920	MCP 54238
52	R11	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836661	MK836921	MCP 54238
53	R12	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836662	MK836922	MCP 54238
54	R14	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836663	MK836923	MCP 54238
55	R15	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836664	MK836924	
56	R17	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836665	MK836925	

57	R19	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836666	MK836926	
58	R2	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836667	MK836927	
59	R21	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836668	MK836928	
60	R22	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836669	MK836929	
61	R3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836670	MK836930	
62	R4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836671	MK836931	
63	R5	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836672	MK836932	
64	R6	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836673	MK836933	
65	R7	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836674	MK836934	
66	R8	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836675	MK836935	
67	R9	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836676	MK836936	
68	PN1	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – Povo Novo	-31.922222 -52.313611	MK836641	MK836901	
69	PN11	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – Povo Novo	-31.922222 -52.313611	MK836642	MK836902	
70	PN2	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – Povo Novo	-31.922222 -52.313611	MK836643	MK836903	MCP 54239
71	PN3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – Povo Novo	-31.922222 -52.313611	MK836644	MK836904	MCP 54239
72	PN4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – Povo Novo	-31.922222 -52.313611	MK836645	MK836905	MCP 54239
73	PN5	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Rio Grande – Povo Novo	-31.922222 -52.313611	MK836646	MK836906	MCP 54239
74	P11	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836611	MK836871	
75	P12	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836612	MK836872	
76	P13	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836613	MK836873	
77	P14	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836614	MK836874	MCP 54240
78	P15	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836615	MK836875	MCP 54240
79	P16	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836616	MK836876	MCP 54240
80	P18	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836617	MK836877	MCP 54240
81	P2	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836618	MK836878	
82	P20	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836619	MK836879	
83	P3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836620	MK836880	
84	P4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836621	MK836881	
85	P5	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836638	MK836898	
86	P6	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836639	MK836899	

87	P7	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836640	MK836900	
88	P8	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MN326793		
89	P22	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MN326794		
90	P23	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411		MK836999	
91	PT1	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836647	MK836907	MCP 54241
92	PT10	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836648	MK836908	MCP 54241
93	PT11	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836649	MK836909	MCP 54241
94	PT12	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836650	MK836910	MCP 54241
95	PT14	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836651	MK836911	
96	PT15	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836652	MK836912	
97	PT16	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836653	MK836913	
98	PT2	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836654	MK836914	
99	PT3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836655	MK836915	
100	PT4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836656	MK836916	
101	PT5	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836657	MK836917	
102	PT6	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836658	MK836918	
103	PT7	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836659	MK836919	
104	J11	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836536	MK836796	MCP 54242
105	J12	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836537	MK836797	MCP 54242
106	J14	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836538	MK836798	MCP 54242
107	J16	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836539	MK836799	MCP 54242
108	J2	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836540	MK836800	
109	J3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836541	MK836801	
110	J4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836542	MK836802	
111	J5	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836543	MK836803	
112	J6	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836544	MK836804	
113	J7	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836545	MK836805	
114	37A	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836485	MK836745	UFRGS 18072
115	37B	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836486	MK836746	UFRGS 18072
116	37C	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836487	MK836747	UFRGS 18072

117	37D	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836488	MK836748	UFRGS 18072
118	37E	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836489	MK836749	UFRGS 18072
119	37F	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836490	MK836750	UFRGS 18072
120	37G	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836491	MK836751	UFRGS 18072
121	37H	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836492	MK836752	UFRGS 18072
122	37I	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836493	MK836753	UFRGS 18072
123	Cri1	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Cristal	-31.078056 -52.038333	KT823671*		
124	Cri2	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Cristal	-31.078056 -52.038333	KT823672*		
125	Cri3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Cristal	-31.078056 -52.038333	KT823673*		
126	Cri4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	Cristal	-31.078056 -52.038333	KT823674*		
127	N13	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050	MK836554	MK836814	MCP 54243
128	N14	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050	MK836555	MK836815	MCP 54243
129	N3	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050	MK836556	MK836816	MCP 54243
130	N4	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050	MK836557	MK836817	
131	N6	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050	MK836558	MK836818	MCP 54243
132	N2	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050		MK836997	
133	N5	<i>C. melanotaenia</i>	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050		MK836998	
134	SM1	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836690	MK836950	MCP 54244
135	SM10	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836691	MK836951	MCP 54244
136	SM11	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836692	MK836952	MCP 54244
137	SM12	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836693	MK836953	MCP 54244
138	SM13	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836694	MK836954	
139	SM14	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836695	MK836955	
140	SM2	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836696	MK836956	
141	SM3	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836697	MK836957	
142	SM4	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836698	MK836958	
143	SM5	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836699	MK836959	
144	SM6	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836700	MK836960	
145	SM7	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836701	MK836961	
146	SM8	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836702	MK836962	

147	SM9	<i>C. intimus</i>	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836703	MK836963	
148	NE1	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836576	MK836836	MCP 54245
149	NE10	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836577	MK836837	MCP 54245
150	NE11	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836578	MK836838	MCP 54245
151	NE12	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836579	MK836839	MCP 54245
152	NE13	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836580	MK836840	
153	NE14	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836581	MK836841	
154	NE15	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836582	MK836842	
155	NE17	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836583	MK836843	
156	NE18	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836584	MK836844	
157	NE19	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836585	MK836845	
158	NE2	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836586	MK836846	
159	NE20	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836587	MK836847	
160	NE21	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836588	MK836848	
161	NE22	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836589	MK836849	
162	NE23	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836590	MK836850	
163	NE3	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836591	MK836851	
164	NE5	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836592	MK836852	
165	NE6	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836593	MK836853	
166	NE7	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836594	MK836854	
167	NE8	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836595	MK836855	
168	NE9	<i>C. nigrovittatus</i>	<i>C. aff. 1 nigrovittatus</i>	Eldorado do Sul	-30.003083 -51.267722	MK836596	MK836856	
169	NA1	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836559	MK836819	
170	NA11	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836560	MK836820	
171	NA12	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836561	MK836821	MCP 54246
172	NA13	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836562	MK836822	MCP 54246
173	NA14	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836563	MK836823	MCP 54246
174	NA15	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836564	MK836824	MCP 54246
175	NA17	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836565	MK836825	
176	NA19	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836566	MK836826	

177	NA2	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836567	MK836827	
178	NA20	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836568	MK836828	
179	NA3	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836569	MK836829	
180	NA4	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836570	MK836830	
181	NA5	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836571	MK836831	
182	NA6	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836572	MK836832	
183	NA7	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836573	MK836833	
184	NA8	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836574	MK836834	
185	NA9	<i>C. nigrovittatus</i>	<i>C. aff. 2 nigrovittatus</i>	Arroio dos Ratos	-30.104485 -51.722630	MK836575	MK836835	
186	NM1	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836597	MK836857	MCP 54247
187	NM10	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836598	MK836858	MCP 54247
188	NM11	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836599	MK836859	MCP 54247
189	NM12	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836600	MK836860	MCP 54247
190	NM13	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836601	MK836861	
191	NM14	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836602	MK836862	
192	NM15	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836603	MK836863	
193	NM2	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836604	MK836864	
194	NM4	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836605	MK836865	
195	NM5	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836606	MK836866	
196	NM6	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836607	MK836867	
197	NM7	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836608	MK836868	
198	NM8	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836609	MK836869	
199	NM9	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836610	MK836870	
200	NM3	<i>C. nigrovittatus</i>	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MN326792		
201	27B	<i>C. notabilis</i>	<i>C. notabilis</i>	Águas claras	-30.095694 -50.849722	MK836483	MK836743	UFRGS 16439
202	27C	<i>C. notabilis</i>	<i>C. notabilis</i>	Águas claras	-30.095694 -50.849722	MK836484	MK836744	UFRGS 16439
203	27A	<i>C. notabilis</i>	<i>C. notabilis</i>	Águas claras	-30.095694 -50.849722		MK836986	UFRGS 16439
204	27D	<i>C. notabilis</i>	<i>C. notabilis</i>	Águas claras	-30.095694 -50.849722		MK836987	UFRGS 16439
205	52C	<i>C. notabilis</i>	<i>C. notabilis</i>	Águas claras	-30.132500 -50.889444	MK836494	MK836754	UFRGS 19629
206	52D	<i>C. notabilis</i>	<i>C. notabilis</i>	Águas claras	-30.132500 -50.889444		MK836988	UFRGS 19629

207	52E	<i>C. notabilis</i>	<i>C. notabilis</i>	Águas claras	-30.132500 -50.889444		MK836989	UFRGS 19629
208	52F	<i>C. notabilis</i>	<i>C. notabilis</i>	Águas claras	-30.132500 -50.889444		MK836990	UFRGS 19629
209	UFRJ	<i>C. notabilis</i>	<i>C. notabilis</i>	Águas claras	-30.096667 -50.851667	KT590068*		
210	SC10	<i>C. feltrini</i>	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722	MK836684	MK836944	
211	SC2	<i>C. feltrini</i>	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722	MK836685	MK836945	
212	SC3	<i>C. feltrini</i>	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722	MK836686	MK836946	
213	SC4	<i>C. feltrini</i>	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722	MK836687	MK836947	
214	SC6	<i>C. feltrini</i>	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722	MK836688	MK836948	
215	SC9	<i>C. feltrini</i>	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722	MK836689	MK836949	
216	SC1	<i>C. feltrini</i>	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722		MK837000	
217	SC5	<i>C. feltrini</i>	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722		MK837001	
218	SC7	<i>C. feltrini</i>	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722		MK837002	
219	SC8	<i>C. feltrini</i>	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722		MK837003	
220	SC11	<i>C. feltrini</i>	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722		MK837004	
221	MT1	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp. 1	Torres	-29.337806 -49.751972	MK836546	MK836806	MCP 54248
222	MT2	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp. 1	Torres	-29.337806 -49.751972	MK836547	MK836807	MCP 54248
223	MT3	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp. 1	Torres	-29.337806 -49.751972	MK836548	MK836808	MCP 54248
224	MX1	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp. 2	Xangri-lá	-29.821833 -50.066389	MK836549	MK836809	MCP 54249
225	MX2	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp. 2	Xangri-lá	-29.821833 -50.066389	MK836550	MK836810	MCP 54249
226	MX3	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp. 2	Xangri-lá	-29.821833 -50.066389	MK836551	MK836811	MCP 54249
227	MX4	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp. 2	Xangri-lá	-29.821833 -50.066389	MK836552	MK836812	MCP 54249
228	MX5	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp. 2	Xangri-lá	-29.821833 -50.066389	MK836553	MK836813	
229	O3	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp. 2	Osório	-29.988889 -50.192222	KT823653*		
230	O4	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp. 2	Osório	-29.988889 -50.192222	KT823654*		
231	O5	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp. 2	Osório	-29.988889 -50.192222	KT823655*		
232	Ci6	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Cidreira	-30.152500 -50.240278	KT823656*		
233	Ci7	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Cidreira	-30.152500 -50.240278	KT823657*		
234	Ci8	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Cidreira	-30.152500 -50.240278	KT823658*		
235	78A	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836495	MK836755	UFRGS 20689
236	78B	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836496	MK836756	UFRGS 20689

237	78C	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836497	MK836757	UFRGS 20689
238	78D	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836498	MK836758	UFRGS 20689
239	78G	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836499	MK836759	UFRGS 20689
240	78J	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836500	MK836760	UFRGS 20689
241	78N	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836501	MK836761	UFRGS 20689
242	78F	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111		MK836991	UFRGS 20689
243	78I	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111		MK836992	UFRGS 20689
244	78O	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111		MK836993	UFRGS 20689
245	81B	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836502	MK836762	UFRGS 20693
246	81C	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836503	MK836763	UFRGS 20693
247	81D	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836504	MK836764	UFRGS 20693
248	81E	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836505	MK836765	UFRGS 20693
249	81G	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836506	MK836766	UFRGS 20693
250	81J	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836507	MK836767	UFRGS 20693
251	81K	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836508	MK836768	UFRGS 20693
252	81N	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836509	MK836769	UFRGS 20693
253	81F	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056		MK836994	UFRGS 20693
254	81L	<i>C. nigrovittatus</i>	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056		MK836995	UFRGS 20693
255	SP1	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836704	MK836964	MCP 54250
256	SP2	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836705	MK836965	MCP 54250
257	SP3	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836706	MK836966	MCP 54250
258	SP4	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836707	MK836967	MCP 54250
259	SP5	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836708	MK836968	
260	SP6	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836709	MK836969	
261	SP7	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836710	MK836970	
262	SP8	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836711	MK836971	
263	SP9	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836712	MK836972	
264	FTM8	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares – Talha Mar	-31.240750 -51.004880	MK836534	MK836794	MCP 54251
265	FTM9	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares – Talha Mar	-31.240750 -51.004880	MK836535	MK836795	MCP 54251
266	T1	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836713	MK836973	

267	T11	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836714	MK836974	MCP 54252
268	T14	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836715	MK836975	MCP 54252
269	T16	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836716	MK836976	MCP 54252
270	T23	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836717	MK836977	MCP 54252
271	T24	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836718	MK836978	
272	T26	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836719	MK836979	
273	T3	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836720	MK836980	
274	T4	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836721	MK836981	
275	T9	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836722	MK836982	
276	T22	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523		MK837005	
277	X3	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	São José do Norte – BujX	-31.527200 -51.291000	MK836723	MK836983	MCP 54253
278	X5	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	São José do Norte – BujX	-31.527200 -51.291000	MK836724	MK836984	MCP 54253
279	X7	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	São José do Norte – BujX	-31.527200 -51.291000	MK836725	MK836985	MCP 54253
280	P41	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836622	MK836882	MCP 54254
281	P410	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836623	MK836883	MCP 54254
282	P411	<i>C. fulgens</i>	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836624	MK836884	MCP 54254
283	P412	<i>C. fulgens</i>	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836625	MK836885	MCP 54254
284	P413	<i>C. fulgens</i>	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836626	MK836886	
285	P414	<i>C. fulgens</i>	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836627	MK836887	
286	P415	<i>C. fulgens</i>	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836628	MK836888	
287	P416	<i>C. fulgens</i>	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836629	MK836889	
288	P42	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836630	MK836890	
289	P43	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836631	MK836891	
290	P44	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836632	MK836892	
291	P45	<i>C. fulgens</i>	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836633	MK836893	
292	P46	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836634	MK836894	
293	P47	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836635	MK836895	
294	P48	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836636	MK836896	
295	P49	<i>C. fulgens</i>	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836637	MK836897	

* Sequences obtained from GenBank. ** MCP = MCT-PUCRS

CAPÍTULO 2

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Colonization history of *Cynopoecilus* (Cyprinodontiformes: Rivulidae) across the western margin of the Patos-Mirim Lagoon System, with the description of a new species

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Colonization history of *Cynopoecilus* (Cyprinodontiformes: Rivulidae) across the western margin of the Patos-Mirim Lagoon System, with the description of a new species

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Abstract

Cynopoecilus Regan, 1912 was recently shown to encompass 8-10 clusters of annual fish that occur in temporary pools of Southern Brazil and Uruguay. Nevertheless, taxonomic boundaries between some of these clusters and their divergence and colonization history were not yet assessed in further detail. This study aims to help in disentangling these complex taxonomic and evolutionary issues through morphological and molecular evaluations of individuals collected on the western margin of the Patos-Mirim Lagoon System, which encompasses a hotspot of annual fish along the Neotropical region. As result, *Cynopoecilus* sp. n., a new species of the *Cynopoecilus melanotaenia* complex is described based on individuals collected in temporary pools located in the Guaíba River and Jacuí River basins, Laguna dos Patos system, Rio Grande do Sul, Brazil. Previously considered as *C. nigrovittatus* and shown to be constituted by two reciprocally monophyletic populations presenting mean distances of 0.012 for the COI marker, the new species is distinguished from all *Cynopoecilus* species by the longest snout length in males, the number of scales in longitudinal series in males, the smallest head length and for the number of gills rakers in the first branchial arch. Also, it is distinguished from all other species of *Cynopoecilus* except *C. feltrini*, by the position of the dorsal-fin origin in females and by the origin of the anal-fin in both sexes. With the results found for the molecular markers, we have been able to suggest a north-south colonization route for the *C. melanotaenia*–*Cynopoecilus* sp. n. complex, that occurred during the last 150 Kya. We also evidenced the action of the Jacuí River as a barrier between *Cynopoecilus* sp. n. and *C. nigrovittatus*, that are recorded in opposite banks of the river.

Key Words: Cynopoecilini, Killifish, Molecular phylogeny, Neotropical, Systematics

INTRODUCTION

Cynopoecilus Regan, 1912 is a genus of killifish of the family Rivulidae, which is endemic from temporary pools in southern Brazil and northeastern Uruguay (Costa, 2002; Costa *et al.*, 2016). This genus is remarkable by having internal fertilization and exhibiting inseminating structures (Costa *et al.*, 2016). Currently, *Cynopoecilus* comprises six species: *C. melanotaenia* Costa 2002, the type species of the genus, *C. notabilis* Ferrer *et al.* 2014, *C. feltrini* Costa *et al.* 2016, *C. fulgens* Costa 2002, *C. intimus* Costa 2002, and *C. nigrovittatus* Costa 2002 (Costa, 2002; Costa *et al.*, 2016; Ferrer *et al.*, 2014). With exception of *C. notabilis*, the remaining species belong to the *C. melanotaenia* complex (Costa *et al.*, 2016). Among these, *C. intimus*, *C. fulgens*, and *C. nigrovittatus* are presented as vulnerable to extinction in local red lists of threatened fauna (Rio Grande do Sul, 2014; ICMBio, 2018).

In a recent molecular phylogenetic study, Garcez *et al.* (2020) detected subdivision of the genus in 8–10 clusters, which are widely incongruent with previous taxonomic boundaries. In fact, this study suggests one invalid taxon (*C. fulgens*), and at least 3–5 putative new species (*Cynopoecilus* sp., *Cynopoecilus* sp. 1, *Cynopoecilus* sp. 2, and possibly *C. aff. nigrovittatus* 1 and *C. aff. nigrovittatus* 2), mostly encompassing populations previously identified as *C. fulgens* and *C. nigrovittatus*. In this case, *C. fulgens* was invalidated because populations from the type locality of the species are mixed with individuals of *C. melanotaenia*. This expanded the distribution of *C. melanotaenia* to the eastern margin of the Patos Lagoon. Concerning *C. nigrovittatus*, besides the type population located at Montenegro (Costa, 2002), there are two

populations previously assigned to the species located on the south bank of the Jacuí river (Volcan *et al.*, 2015). However, these three populations of *C. nigrovittatus* revealed to be polyphyletic and were subdivided into three independent lineages by Garcez *et al.* (2020), which provisionally attributed to the two new populations the status of putative new species.

Although Garcez *et al.* (2020) evidenced that vicariance is an important diversification mechanism of *Cynopoecilus*, it also suggested the occurrence of isolated and recent dispersal events even across large water bodies, as the Patos-Mirim Lagoon System (PMLS). The area occupied by PMLS was intensely influenced by geomorphological events related to sea level variations that occurred during the last 400 Kya (Villwock and Tomazelli, 2007). In fact, the onset of the formation of the Patos Lagoon seems to have occurred about 325 Kya, during the so called “Second Depositional Event of the Multiple Barrier System”, whereas its final implementation seems to have occurred about 120 Kya, during the so called “Third Depositional Event of the Multiple Barrier System” (Villwock and Tomazelli, 2007). This large water body strongly influenced the structure and the evolution of *Austrolebias wolterstorffi*, a species that frequently co-occurs with different *Cynopoecilus* species (Garcez *et al.*, in preparation). In fact, *A. wolterstorffi* seems to be subdivided in two main clusters, whose spatial distribution is restricted to different margins of the PMLS, and that evidenced a north-south colonization pattern occurring during the last 325 Kya (Garcez *et al.*, 2018). Although Garcez *et al.* (2020) evidenced a derived lineage encompassing *C. melanotaenia*, *C. aff. nigrovittatus* 1 and *C. aff. nigrovittatus* 2, that is mostly isolated to the southern margin of the Jacuí River and the western margins of the Patos-Mirim Lagoon System, the spatio-temporal pattern of diversification of this clade remains yet to be evaluated.

Thus, this study aims to help in disentangling the complex taxonomic and evolutionary issues related to the species of *Cynopoecilus* that mostly occur across the western margin of the PMLS. To fulfil these goals, we first carried out detailed morphological inspections in *C. aff. nigrovittatus* 1 and *C. aff. nigrovittatus* 2, two cases in which taxonomic identity should be reviewed. We also performed molecular analyses to address the spatio-temporal pattern of colonization of the major lineage to which these two clusters are assigned. As results of molecular and morphological analyses we herein describe a new species of annual fish from southern Brazil which is closely related to the *C. melanotaenia*. The new species is diagnosed by morphology patterns, genetic distances and through phylogenetic tree generated by the analysis of a segment of the mitochondrial cytochrome c oxidase subunit I (COI) gene and the nuclear recombination activating protein 1 (RAG1) gene. We also evidenced a north to south colonization route for the *C. melanotaenia*–*Cynopoecilus* sp. n. complex, that occurred during the last 150 Kya.

MATERIALS AND METHODS

Collections and initial processing of the sampled specimens were accomplished as described in Garcez *et al.* (2020). Morphological characters were obtained from specimens just after euthanasia with an overdose of 3,000 mg/L of eugenol anesthetic, after transferring them to 70% ethanol. Specimens used in the molecular analysis were fixed in 98% ethanol. DNA extractions, amplification of the mitochondrial cytochrome c oxidase subunit I (COI) gene and recombination activating protein 1 (RAG1) and sequencing were performed as described in Garcez *et al.* (2020). Furthermore, sequences of the nuclear marker Ectoderm-neural cortex protein 1 (ENC1) were obtained as described in Garcez *et al.* (in preparation). The list of specimens and their

respective GenBank accession numbers appear in Table S1. 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).

Species delimitation and description

The methodology employed for species delimitation follows an integrative approach (e.g., Costa *et al.*, 2016), using a morphological character-based method and a molecular tree-and-distance-based method.

Morphology-based evaluations

The descriptions of the colour patterns were based on photographs of live specimens, photographed in the field in both sides of males and females, before fixing. Morphometric data were taken following Costa (1995) and the measurements are expressed as a percentage of the standard length (SL), except for the measures related to head morphology, which are recorded as a percentage of head length. About meristic data, fin-ray counts include all elements. Osteological preparations were made on specimens clarified and stained (C&S) according to the method described in Taylor and Van Dyke (1985). Frontal squamation nomenclature follows Hoedeman (1958) and the cephalic neuromast series Costa (2006).

Molecular-based evaluations

In the tree-based approach, concatenated trees were generated through the analysis of 1,182 bp of the COI gene and 562 bp of the RAG1 gene using Bayesian inferences (BI), to evaluate the presence of reciprocal monophyly. This analysis was 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. The phylogenetic trees were further visualized and edited in FigTree 1.4.3 (Rambaut and Drummond, 2009). Genetic distances, herein used to illustrate genetic diversity among taxa based in COI gene, were measured in MEGA 7.0 with the Kimura 2-Parameters (K2P) evolutionary model (Kimura, 1980).

Population structure

The relationships among haplotypes of COI were inferred from networks generated by median-joining in the Network v.4.510 software (Bandelt *et al.*, 1999). The levels of genetic differentiation among populations of the same species were measured by the fixation index (FST) in Arlequin 3.5 (Excoffier and Lischer, 2010)

Evolutionary scenario

A chronophylogenetic analysis was performed under a Bayesian Analysis (BA) using BEAST 1.10.4 (Suchard *et al.*, 2018), on CIPRES with the use of the model selected for each marker by BIC in jModelTest. For this analysis, we employed a multi-locus strategy, using COI, ENC1 and RAG1 sequences characterized for *C. melanotaenia*, *C. aff. nigrovittatus* 1, *C. aff. nigrovittatus* 2 and *C. nigrovittatus*, using sequences of *A. wolterstorffi* sampled in both, the east and west margins of the Patos Lagoon as outgroup. The dating for the divergence between eastern and western populations of *A. wolterstorffi* first established by Garcez *et al.* (2018) to 0.325 Mya (± 0.0125) was used as a prior to calibrate the molecular clock, together with the rates estimated for COI,

ENC and RAG1 using the same calibration system. The analysis was carried out under unlinked substitution and clock models, but with linked coalescent trees, under both, a strict and a longnormal relaxed uncorrelated molecular clock. The MCMC analysis was run for 500 million generations, with trees and parameters sampled every 10,000 iterations, and burning the 10% initial results. Results were first visualized in Tracer 1.6 (Rambaut *et al.*, 2014) to evaluate convergence and ESS values, and to compare performance of both molecular clock models by AICM (Baele *et al.*, 2012), with standard deviations calculated from 100 bootstrap replications. The maximum clade probability tree was constructed by TreeAnnotator 1.8.4 (Drummond and Rambaut, 2007), and then visualized and edited in FigTree.

Extended Bayesian Skyline Plot (EBSP) analyses was performed in BEAST 1.10.4 (Suchard *et al.*, 2018) to evaluate demographic alterations of population sizes over time using the three molecular markers. This analysis was performed using unlinked substitution models, as selected by BIC in jModelTest. Priors for the unlinked clock models were established based on the mean and stdev of the ucl.d.means obtained for each gene in the chronophylogenetic analysis described above, under the clock model chosen by the AICM test performed in Tracer for the same analysis. Scale factors of the population models were proportional to effective population sizes of each marker. The MCMC analysis was run for 500 million generations, with trees and parameters sampled every 5,000 iterations, and burning the 10% initial results. Results were first visualized in Tracer 1.6 (Rambaut *et al.*, 2014) to evaluate convergence and ESS values.

The colonization route was determined using an ancestral state character reconstruction analysis under a symmetric discrete trait substitution model in a BA implemented in BEAST v. 1.10.4. This analysis was performed using unlinked substitution models for COI, ENC1 and RAG1, as selected by BIC in jModelTest.

Priors for the unlinked clock models and clock models were the same employed in the EBSF analysis described above, and tree priors were set to a constant size coalescent. The MCMC analysis was run for 500 million generations, with trees and parameters sampled every 10,000 iterations, and burning the 10% initial results. Results were first visualized in Tracer, and then the maximum clade probability tree was constructed by TreeAnnotator. This tree was used to visualize the spatial diffusion over time, as performed by SPREAD (Bielejac *et al.*, 2011), with time intervals compatible with the results of the chronophylogenetic analysis. The location of ancestral area was plotted to the map at three specific time intervals (approximately 150 Kya, 75 Kya, 33 Kya and present).

RESULTS

Species delimitation by molecular analyses

Phylogenetic analysis

The phylogenetic tree generated through BI (Figure 1) subdivided all sampled specimens of *Cynopoecilus* into ten clusters. Except for *C. notabilis* and *C. intimus*, these clusters branched out to divide into two main clades: Clade A, encompassing populations inhabiting drainages located at the western and/or south margins of the Laguna dos Patos and the Jacuí River; Clade B, grouping populations sampled along drainages located at eastern and/or north margins of these large water bodies. Within clade A, the two populations collected at the southern margin of the Jacuí River, that were previously assigned to *C. nigrovittatus* (Volcan *et al.*, 2015), but later identified as *C. aff. nigrovittatus* 1 and *C. aff. nigrovittatus* 2 by Garcez *et al.* (2020), were recovered as reciprocally monophyletic lineages. The clade composed by these two populations is

closer to *C. melanotaenia* than to *C. nigrovittatus*.

Genetic distances

Considering the subdivision into ten lineages, intra-lineages COI distances varied from 0% to 2.3%, with a mean of 0.23%. Conversely, inter-lineage COI divergences varied from 1% to 24.9%, with a mean of 7.9%. This revealed the absence of a barcoding gap for the entire genus. The lower inter-lineages COI distances were recovered in the comparison between *C. aff. nigrovittatus* 1 and *C. aff. nigrovittatus* 2, raising the possibility that they can constitute two lineages within the same species. When both lineages are considered populations of a single species, maximum intra-lineages COI distances changes to 2.3%, whereas the minimum inter-lineages distances changes to 1.4%, leaving a barcoding gap of 0.9%.

Species delimitation by morphological analyses

Forty-five and 41 individuals from the two populations *Cynopoecilus* sampled at drainages located at the southern margin of the Jacuí River [of the populations of Eldorado do Sul – RS, and Arroio dos Ratos – RS, previously referred as *C. aff. nigrovittatus* 1 and *C. aff. nigrovittatus* 2 (Garcez *et al.*, 2020), respectively] were evaluated in regard to colour patterns, morphometric, meristic and osteological data. As no difference could be encountered between them, they are now considered a single species, that is described below.

***Cynopoecilus* sp. n. (Figures 2–4)**

Holotype

Male, 35.4 mm SL, Brazil, Rio Grande do Sul State, Eldorado do Sul municipality,

251 Laguna dos Patos system, Guaíba river basin, temporary pool in the floodplains of Rio
252 Jacuí, close to BR-116 highway (30°00'13" S, 51°16'06" W), Oct. 2020, D. K Garcez,
253 M. V. Volcan, G. R. Ozório & C. Barbosa.

254

255 **Paratype**

256 Nine males, 29.4–39.7 mm SL (2 C&S), collected with holotype. Sixteen females, 23.0–
257 27.6 mm SL (2 C&S), collected with holotype. MCT-PUCRS 48193, 5 males, 26.9–
258 33.4 mm SL and 14 females 23.3–30.5 mm SL, same locality of holotype, Aug. 2012,
259 L. E. K. Lanés, M. V. Volcan & G. M. Wallwitz. Twenty-one females (2 C&S), 15.6–
260 32.2 mm SL and 20 males (2 C&S), 22.0–41.1 mm SL, collected in temporary pool in
261 the floodplains of lower Jacuí river basin, Arroio dos Ratos municipality, close to BR–
262 290 highway (30°06'16" S, 51°43'21" W), October 20, 2020, D. K Garcez, M. V.
263 Volcan, G. R. Ozório and C. Barbosa.

264

265 **Diagnosis**

266 *Cynopoecilus* sp. n. can be distinguished from all congeners by fewer gill rakers in the
267 first branchial arch (2+5–7 vs. 2–3+8–9 in remaining species). It is distinguished from
268 all congeners by lower head length in males (22.2–25.9% SL vs. 27.6–30.2% SL in *C.*
269 *feltrini*, 28–31.8% SL in *C. nigrovittatus*, 28.2–31.5% SL in *C. melanotaenia*, 28.2–
270 30.4% SL in *C. notabilis*, 28.6–30.9% SL in *C. intimus*, and 26.4–28.4% SL in *C.*
271 *fulgens*) and females (20.8–24.3% SL vs. 27.2–29.2% SL in *C. fulgens*, 27.2–29.8% SL
272 in *C. feltrini*, 27.6–31.2% SL in *C. nigrovittatus*, 27.8–31.4% SL in *C. melanotaenia*,
273 27.9–31% SL in *C. notabilis*, and 27.4–30% SL in *C. intimus*). It is also distinguished
274 from all congeners by the larger snout length in males (16.3–27.2% SL vs. 13.1–14.2%
275 SL in *C. fulgens*, 12.9–14.6% SL in *C. intimus*, 12.7–14.7% SL in *C. feltrini*, 12.3–15%

276 SL in *C. melanotaenia*, 12.3–14.2% SL in *C. nigrovittatus*, and 7.6–9.6% SL in *C.*
277 *notabilis*). Females of this species can be distinguished from all congeners by having a
278 pattern of small light brown or dark brown spots on the dorsal and anal fins (vs. no
279 similar pattern). It is also distinguished from all other *Cynopoecilus* species in having
280 brown spots on the dorsolateral scales that extend from the head to the caudal peduncle
281 (vs. no similar pattern). Additionally, it is distinguished from all other species of
282 *Cynopoecilus* except *C. feltrini*, by having the dorsal-fin origin at a vertical position
283 through the urogenital papilla in females (vs. dorsal-fin origin at a vertical position
284 through the anus) and for presenting the anal-fin origin along vertical between pleural
285 ribs 10 and 13 in males (vs. anal-fin origin between pleural ribs 7 and 8) and between 13
286 and 14 in females (vs. anal-fin origin between 7 and 8).

287 *Cynopoecilus* sp. n. is distinguished from *C. melanotaenia* by presenting more
288 scales in the longitudinal series (28–29 vs. 25–27) and the pectoral-fin rays (13–14 vs.
289 12–13), smaller caudal peduncle depth (11.6–13.6% SL vs. 13.5–15.4% SL) and pelvic-
290 fin length (3.5–6.9% SL vs. 6.8–7.3% SL), and larger eye diameter (36.4–43.5% HL vs.
291 29.6–36.1% HL). Pectoral fin with posterior margin reaching the pelvic-fin base in
292 males (vs. posterior pelvic-fin) and pelvic fin reaching the space between the anus and
293 the urogenital region in males (vs. urogenital papilla) and the anus in females (vs.
294 urogenital papilla).

295 It differs from *C. nigrovittatus* by having the pelvic-fin margin reaching the
296 space between the anus and the urogenital region in males (vs. urogenital papilla) and
297 the anus in females (vs. urogenital papilla), by presenting more scales in the longitudinal
298 series (28–29 vs. 26–28) and by having greater head depth in females (76.2–96.6% HL
299 vs. 70.8–76.1% HL).

300 It is distinguished from *C. intimus* by presenting dorsal and anal fin with dark

301 brown spots and caudal fin with light brown spots in males and dorsal, caudal and anal
302 fins with small light brown or dark brown spots in females (vs. dark brown blotches on
303 dorsal and caudal fins, faint grey spots on anal fin). Pectoral fin with posterior margin
304 reaching the pelvic-fin base in males (vs. posterior pelvic-fin). More scales in the
305 longitudinal series (28–29 vs. 25–26) and largest eye diameter in females (37.2–49.6%
306 HL vs. 35.2–37.3% HL).

307 It differs from *C. fulgens* by the lower body depth in males (22.4–27.4% SL vs.
308 27.5–29.5% SL), larger eye diameter in males (36.4–43.5% HL vs. 31.5–33.8% HL)
309 and females (37.2–49.6% HL vs. 33.1–35.9% HL) and by the pelvic fin reaching the
310 anus in females (vs. urogenital papilla).

311 *Cynopoecilus* sp. n. differs from *C. feltrini* by the lower body depth in males
312 (22.4–27.4% SL vs. 27.4–32% SL), by presenting fewer rays in the caudal (26–29 vs.
313 29–32) and anal fin (22–24 vs. 25–27), by the dorsal-fin origin in males (vertical
314 between the anus and the urogenital papilla vs. pelvic anterior), by having more scales
315 in the longitudinal series (28–29 vs. 27–28) and by presenting frontal squamation E or
316 F-patterned (vs. E or D pattern).

317 Finally, it differs from *C. notabilis* in several characters: longer pre-dorsal length
318 in males (52.8–60% SL vs. 47–50.4% SL) and females (56.6–64.2% SL vs. 48.8–55.1%
319 SL); smaller dorsal-fin base length in males (23.8–31% SL vs. 33.9–36.4% SL) and
320 females (21–28.3% SL vs. 29.4–34.9% SL); smaller pectoral-fin length in males (16.2–
321 21.1% SL vs. 21.8–26.7% SL); smaller head depth in males (65.6–88.2% HL vs. 15.9–
322 18.5% HL) and females (76.2–96.6% HL vs. 15.5–18.9% HL); smaller head width in
323 males (66.3–85.1% HL vs. 16.2–19.1% HL) and females (71–87.8% HL vs. 17.7–20.1%
324 HL); longer lower jaw length in males (16.9–29.7% HL vs. 10.1–11.5% HL) and
325 females (12.2–25.4% HL vs. 9.1–12.1% HL); longer eye diameter in males (36.4–

43.5% HL vs. 8.7–11% HL) and females (37.2–49.6% HL vs. 9.4–12% HL); fewer rays on the dorsal fin (17–19 vs. 20–23); pectoral fin with posterior margin reaching vertical through the pelvic-fin base in males (vs. urogenital papilla); pelvic fin pointed reaching the space between the anus and the urogenital region in males (vs. urogenital papilla); dorsal-fin origin in a vertical position between the anus and the urogenital region (vs. pelvic fin); fewer scales in the longitudinal series (28–29 vs. 29–31) and around the caudal peduncle (11–14 vs. 16); frontal squamation E or F-patterned (vs. E pattern).

Description

Morphometric data is shown in Table 1. Males larger than females, largest male examined 41.1 mm SL, largest female 32.2 mm SL.

External morphology

See Figure 2 for general external morphology. Body elongated and slightly compressed. Longest body depth on a vertical through pelvic-fin base. Dorsal profile on lateral view concave from snout to vertical through anterior margin of the operculum and convex from this point to the posterior end of the dorsal-fin base, approximately straight on the caudal peduncle. Ventral profile on lateral view between the lower jaw and end of anal-fin base smoothly convex to approximately straight in male, convex in female, and approximately straight on the caudal peduncle of both sexes.

Pectoral fin elliptical with the posterior margin reaching vertical through pelvic-fin base in both sexes. Pectoral-fin rays 13–14. Pelvic fin pointed, reaching the space between the anus and the urogenital region in males and the anus in females. Pelvic-fin rays 6, except one male with only a single atrophied right pelvic-fin. Pelvic fin separated by a small interspace. Urogenital papilla attached to the anterior margin of the

anal fin. Dorsal fin rays 17–19. Dorsal fin in males larger than females. Dorsal pointed and long in male, slightly pointed in female. Dorsal-fin origin in a vertical position between the anus and the urogenital region. Anal fin rays 22–24. Anal fin in males long and pointed, slightly rounded in females. Caudal fin rays 26–29. Caudal fin rounded to subtruncate.

Squamation and contact organs

Scales large, cycloid. Ctenii-like contact organs on caudal peduncle. No contact organs on the pectoral-fin of males. Frontal squamation E or F-patterned. 15% of the individuals presented E scales medially overlapped. Longitudinal series of scales 28–29, transverse series of scales 8–11, and scale rows around caudal peduncle 11–14.

Neuromasts

Cephalic neuromasts: supraorbital 10–16, parietal 1, anterior rostral 1, posterior rostral 1, infraorbital 14–29, preorbital 1–4, otic 1–3, postotic 1–4, supratemporal 1–2, median opercular 1–5, ventral opercular 2–6, preopercular 11–27, mandibular 5–11, lateral mandibular 2–7, paramandibular 1, and peduncle 1–7.

Osteology

Dorsal-fin origin between neural spines of vertebrae 10 and 13 in males and through the neural spines of vertebrae 10 and 12 in females. Origin of anal fin at a vertical position through the pleural ribs 10 and 13 in males and through the pleural ribs 13 and 14 in females.

Six branchiostegal rays. Dermosphenotic ossification absent. Total number of vertebrae 27–30. Gill rakers in first branchial arch 2+5–7. Basihyal subtriangular, width

about 35-50% of length; basihyal cartilage about 30-40% of total basihyal length. One tooth on the second pharyngobranchial. Vomerine teeth absent. Hypurals forming single plate.

Colour in life

Males (Figures 2 and 3): Two longitudinal dark brown stripes along lateral surface of the body, one extending from the lower jaw to the caudal fin base and the other from the pectoral-fin base across belly to the posterior portion of anal-fin base, anteriorly overlapped by small pale light blue spots. Body light brown above the lateral stripe and light yellowish between the two longitudinal black stripes and ventrally on belly. On the body side and opercular region, the stripe is overlapped by a longitudinal series of spots, greenish blue on the opercle and bright blue on the body, each spot on the center of each scale. Dark brown spots on the dorsolateral scales extending from the head to the caudal peduncle. Iris yellow, with a vertical dark brownish bar. Dorsal fin yellowish hyaline with dark brown spots extending from the base to the second third of the fin. Anal fin hyaline with dark brown spots limited to the posterobasal region. Caudal fin pale reddish hyaline with light brown spots in the basal half. Pectoral and pelvic fins hyaline.

Females (Figures 2 and 4): Colour pattern similar to that described for males, but with brown lateral longitudinal stripes, overlapped by a longitudinal series of scales bright greenish blue on the opercle and bright blue on the body. Sometimes, the longitudinal stripe of the body is interrupted, forming dark brown spots. All fins are hyalines. Dorsal, caudal and anal fins with small light brown or dark brown spots.

Distribution

Cynopoecilus sp. n. is registered for temporary pools located on two regions of the

floodplains of the lower course of the Jacuí river, southern Brazil (Figure 5). The type locality (Figure 6) is located in the municipality of Eldorado do Sul, Guaíba river basin. The second population is located in the municipality of Arroio dos Ratos, distant about 45 km from the type locality.

Etymology

Named in honour of one of the greatest environmentalists from Rio Grande do Sul, a pioneer, and a true icon of the ecological cause in Brazil.

Ecological notes

Cynopoecilus sp. n. was found only in two isolated temporary wetlands. The type locality is located close to BR-116 highway, on the floodplains of the Jacuí river, Guaíba river basin. The area presented cloudy waters, with signs of eutrophication, probably due to the sewage discharge. The other population is also located close to BR-290 highway, on the floodplains of the Ratos stream, lower Jacuí basin. It presented clear waters, with a small fragment of shrubs in the surroundings. *Cynopoecilus* sp. n. was collected with the annual fish *A. wolterstorffi* Ahl (1924) and *A. adloffii* Ahl (1922), and with the non-annual fish *Astyanax laticeps* Cope (1894), *Hyphessobrycon luetkenii* Boulenger (1887), *Callichthys callichthys* Linnaeus (1758) and *Hisonotus* sp.

Conservation

The type locality of *Cynopoecilus* sp. n. occurs in temporary wetlands that are threatened by the advance of urbanization and the consequent discharge of effluents. When the species was collected for the first time, co-occurrence was recorded with *A. wolterstorffi* and *A. adloffii*. Nevertheless, in later collections (2016 to 2020), we were

not able to find these last two species again, and only *Cynopoecilus* sp. n. was collected. The other population is found on the banks of the BR-290 highway and suffers from mining that occurs a few meters from the wetland and from the cultivation of rice and soybeans in its drains.

Analyses of population structure

Each of the populations of *Cynopoecilus* sp. n. was recovered as independent haplogroups in the COI network (Figure 7). These were interconnected for at least five mutational steps, without a single case of haplotype sharing between the two populations. Conversely, the populations of *C. melanotaenia* presented a general star-like pattern and are separated from *Cynopoecilus* sp. n. by at least nine mutational steps.

Most of the pairwise comparisons recovered for COI showed significant genetic structure and the F_{ST} values varied from 0 to 1.00 (in the comparison between populations of *C. melanotaenia*), from 0.908 to 0.979 (between populations of *C. melanotaenia* and *Cynopoecilus* sp. n.) and it was 0.865 between the two populations of *Cynopoecilus* sp. n.

Spatio-temporal evolutionary scenario

The chrono-phylogenetic analysis performed with the three molecular markers inferred that the divergence between *Cynopoecilus* sp. n. and *C. nigrovittatus* occurred about 764 MKya (95% HPD of 570-967) (Figure 8). The divergence of the new species and *C. melanotaenia* seems to have occurred more recently, about 344 Kya (95%HPD of 238-451). The Most Recent Common Ancestor (MRCA) of *Cynopoecilus* sp. n. was inferred to 199 Kya, which coincides with the divergence time between the two populations of lineages of the new species. (Figure 8). Finally, the diversification of the

Eldorado do Sul and Arroio dos Ratos *Cynopoecilus* sp. n. lineages was dated to about 119 Kya and 0.035 Kya, respectively.

The EBSP analysis reconstructed with the total set of sequences of *C. melanotaenia* and for both lineages of *Cynopoecilus* sp. n. did not reject the hypothesis of constant population sizes, since the 95%HPD did not exclude zero.

The colonization route of the clade encompassing *C. nigrovittatus*, *C. melanotaenia* and *Cynopoecilus* sp. n. clearly suggest a north-south colonization route (Figure 9). In this sense, the MRCA of the lineage seems to have occurred in a restricted region near the area where the Jacuí River flows into the Patos Lagoon until about 150 Kya (Figure 9A). Only around 75 Kya, the lineage seems to have expanded its distribution to the Southern of the Patos Lagoon (Figure 9B). Around 33 Kya, areas on the Southeastern margin of the Patos Lagoon and the Northeastern margin of the Mirim Lagoon seems to have been occupied (Figure 9C). Uruguayan areas were only occupied about 21 Kya, and since then, Uruguay and Brazil seems to have frequently exchanged migrants, in both directions (Figure 9D).

DISCUSSION

Both morphological and molecular analyses support here a new species of *Cynopoecilus*, herein identified as *Cynopoecilus* sp. n. The two populations located on the south bank of the Jacuí River here assigned to the new species were previously identified as *C. nigrovittatus* by Volcan *et al.* (2015). However, Garcez *et al.* (2020) found that *C. nigrovittatus* is restricted to the north bank of the Jacuí River, suggesting that the two populations on the opposite bank encompass two lineages that could represent one or two new species (provisionally named *C. aff. nigrovittatus* 1 and *C. aff. nigrovittatus* 2). In this study, we confirmed the reciprocal monophyly of both lineages,

and the moderate distances presented between them (minimum of 1%). Besides, we also supported the sister group relationships of both lineages, which was not recovered by Garcez *et al.* (2020). As none morphological difference could be found for individuals collected in both populations, we confirmed here they encompass a single species, with a deep population structure.

The known localities of *Cynopoecilus* sp. n. are situated in the south bank of the Jacuí River, in the municipalities of Eldorado do Sul and Arroio dos Ratos. Conversely, *C. nigrovittatus* is encountered in the north bank of the same River, in the municipality of Montenegro. Despite the geographical proximity between these two species (about 40 km), morphological, phylogenetic, and genetic distance analyses showed that they are not closely related. This indicates that the Jacuí River has been a historical barrier for gene flow among these species, as also suggested by Garcez *et al.* (2020). Evidence of water bodies acting as barriers to gene flow have also been found for other Neotropical annual killifish species (Barbosa *et al.*, 2020; Costa *et al.*, 2017; Fernandes *et al.*, 2021; Garcez *et al.*, 2018) and African annual killifish (Bartáková *et al.*, 2013, 2015). Nevertheless, even for species or populations inhabiting the same area, there may be specificities related to the spatio-temporal evolutionary pattern. In this case, whereas the divergence between *C. nigrovittatus* and *Cynopoecilus* sp. n. seems to have occurred about 764 Kya, with a strong influence of the Jacuí River, the diversification of *A. wolterstorffi* seems to have occurred in associated with the formation of the Patos Lagoon (Garcez *et al.*, 2018), whose onset was dated to approximately 325 Kya (Villwock and Tomazelli, 2007).

According to the spatial diffusion phylogeographic analysis, the vicariance event associated with the allopatric speciation of *Cynopoecilus* sp. n. and *C. nigrovittatus* seems to have been followed by dispersion and new colonisations to the south of these

ancestral areas, following a north-to-south diversification. In agreement with this, the topology of the tree and the genetic distances suggest a close relationship between *Cynopoecilus* sp. n. and *C. melanotaenia*, both of which occur to the south of the Jacuí River. A north-to-south diversification was also detected by Garcez *et al.* (2018) for *A. wolterstorffi*, for the lineages distributed on both margins of the Patos Lagoon. Interestingly, although the divergence between *Cynopoecilus* sp. n. and *C. melanotaenia* was dated to about 344 Kya, areas to the south of the Patos Lagoon seems to have been occupied only in the last 150-75 Kya. This period spans the same limits presented for the diversification of the lineages of *A. wolterstorffi* inhabiting more southern distributions (Garcez *et al.*, 2018), raising the possibility of concomitant dispersion waves.

The diversification of *Cynopoecilus* sp. n. occurred about 199 Kya, which marks the divergence between both lineages of the species. This time coincides with the diversification of *C. melanotaenia*, which occurred about 189 Kya. Interestingly, the last species greatly expanded the distribution of the genus, occupying regions to the South-East of the Patos Lagoon and the North-East of the Mirim Lagoon about 33 Kya, and later expanding to Uruguayan areas, whereas the first seems to have remained restricted to ancestral areas. Nonetheless, this contrasting distribution pattern is associated with quite similar population structures: despite *Cynopoecilus* sp. n. presents only two known populations that are geographically close (only about 45 Km apart) but strongly structured ($F_{st} = 0.948$), *C. melanotaenia* presents more than 35 populations characterized so far, that can be as distant as 390 km, and also present high levels of genetic differentiation (mean $F_{st} = 1.000$). These similar patterns of structuring can be related to similar adaptations encountered in these species.

Concerning specific adaptations, although *Cynopoecilus* sp. n. and *C.*

melanotaenia are phylogenetically closer between each other than they are to any other species of the genus, they present important morphological differences. In fact, these species differ by snout length in males; scales in the longitudinal series in males; head length; caudal peduncle depth in males; pelvic-fin length in males; number of pectoral-fin ray; eye diameter; pattern of colouration and spots of dorsal, caudal and anal fins in females and by the number of gills rakers in the first branchial arch. Nevertheless, the putative influence of these differences in the dispersion capabilities of each of these species remains yet to be evaluated.

Cynopoecilus sp. n. can also be distinguished from all the other species of *Cynopoecilus*. In fact, although individuals of the new species were first identified as *C. nigrovittatus* (Volcan *et al.*, 2015), these species can be distinguished by the head length, head depth in females, snout length in males, pelvic fin reach, anal-fin origin and by the number of gills rakers in the first branchial arch. Nevertheless, colour patterns exhibited by males of *Cynopoecilus* sp. n. and *C. nigrovittatus* are similar. This agrees with the observation presented by Costa *et al.* (2016), according to which species of the *C. melanotaenia* complex form a morphologically homogeneous group (Figures 10 and 11), with all species highly differing from another congener, *C. notabilis*.

Cynopoecilus sp. n. is also distinguished from all other species of *Cynopoecilus* except *C. feltrini*, by the origin of the dorsal-fin in females and by the origin of the anal-fin in both sexes. According to (Costa *et al.*, 2016), *C. feltrini* is distinguished from all other species of the *C. melanotaenia* complex by having frontal E-scales medially overlapped (vs. separated by interspace). However, 15% of the analyzed specimens of *Cynopoecilus* sp. n. have E-scales medially overlapped also, a pattern that is not frequent in other species of the *C. melanotaenia* complex. Nevertheless, the orangish red branchiostegal region encountered in males of *C. feltrini* (vs. hyaline in

Cynopoecilus sp. n.), and its dorsum with few dark brown spots above the opercular region (vs. dark brown spots over most region between the head and the caudal peduncle in *Cynopoecilus* sp. n.) easily distinguished both species.

Cynopoecilus intimus is another species that is recorded in the Jacuí River basin, for drainages about 213 km from the populations of *Cynopoecilus* sp. n. Both species are distinguished by the color pattern of the fins in males, because *Cynopoecilus* sp. n. has dorsal and anal fin with dark brown spots and caudal fin with light brown spots (vs. dark brown blotches on dorsal and caudal fins, faint grey spots on anal fin). In addition, the phylogenetic analysis consistently indicates that *C. intimus* encompasses an early offshoot within the *C. melanotaenia* complex, that differs from *Cynopoecilus* sp. n. by a minimum of 2.9% in COI sequences.

Specifically in regard to drainages associated with the Jacuí River, this study adds in characterizing it as a hotspot for the diversity of annual fish in the Neotropics, highlighting the need of conserving its temporary wetlands. In fact, with this study, there are registers of three species of *Cynopoecilus* to this region: *C. intimus*, occurring in the upper course of the river; and *C. nigrovittatus* and *Cynopoecilus* sp. n., in the lower course of the river, but on opposite banks. In addition, five other species of annual fish are recorded in the Jacuí river basin: *A. adloffii*, *A. wolterstorffi*, *A. litzi*, *A. cyaneus*, *A. paucisquama* (Volcan *et al.*, 2015). All these species are considered threatened with extinction (Rio Grande do Sul, 2014; ICMBio, 2018), mainly due to the loss and drainage of temporary wetlands for rice and soybean crops, or by the advance of the urban area. This is also the case for *Cynopoecilus* sp. n., which presents a restricted and impacted area of occurrence and should be included in future lists of threatened fauna.

In conclusion, the use of molecular markers and morphological inspections

added resolution to the evolutionary scenario of *Cynopoecilus* and confirmed the existence of a new species, as previously suggested by Garcez *et al.* (2020) in a molecular approach. It also evidenced the subdivision of the new species in two lineages, which should since now guarantee their individual conservation. This discovery endorses the argument that the current annual fish diversity along the PMLS is underestimated (Garcez *et al.*, 2018, 2020; Volcan *et al.*, 2015). It also provides evidence that this diversity may have evolved through some common and other alternative mechanisms, that certainly lead to the establishment of important hotspots that need urgent conservation efforts.

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726 TABLES

727 **Table 1** Morphometric data for the holotype and paratypes of *Cynopoecilus* sp. n.

Standard length (mm)	Holotype	Paratypes	
	Male	Males (n=34)	Females (n=51)
	35.4	22.0–41.1	15.6–32.2
Percentages of standard length			
Body depth	27.2	22.4–27.4	21.8–30.9
Caudal peduncle depth	13.6	11.6–13.6	10.8–14.1
Pre-dorsal length	56.2	52.8–60	56.6–64.2
Pre-pelvic length	45.8	42.8–51	47.5–54.3
Dorsal-fin base length	26.9	23.8–31	21–28.3
Anal-fin base length	25.2	20.4–28.4	17.3–23
Caudal-fin base length	27.3	26.3–36.1	23.9–32.5
Pectoral-fin length	18.1	16.2–21.1	16.5–20.4
Pelvic-fin length	5.5	3.5–6.9	4.7–8.2
Head length	24.2	22.2–25.9	20.8–24.3
Percentages of head length			
Head depth	71.6	65.6–88.2	76.2–96.6
Head width	74.5	66.3–85.1	71–87.8
Snout length	24.2	16.3–27.2	11.7–27.8
Lower jaw length	24.8	16.9–29.7	12.2–25.4
Eye diameter	38.6	36.4–43.5	37.2–49.6

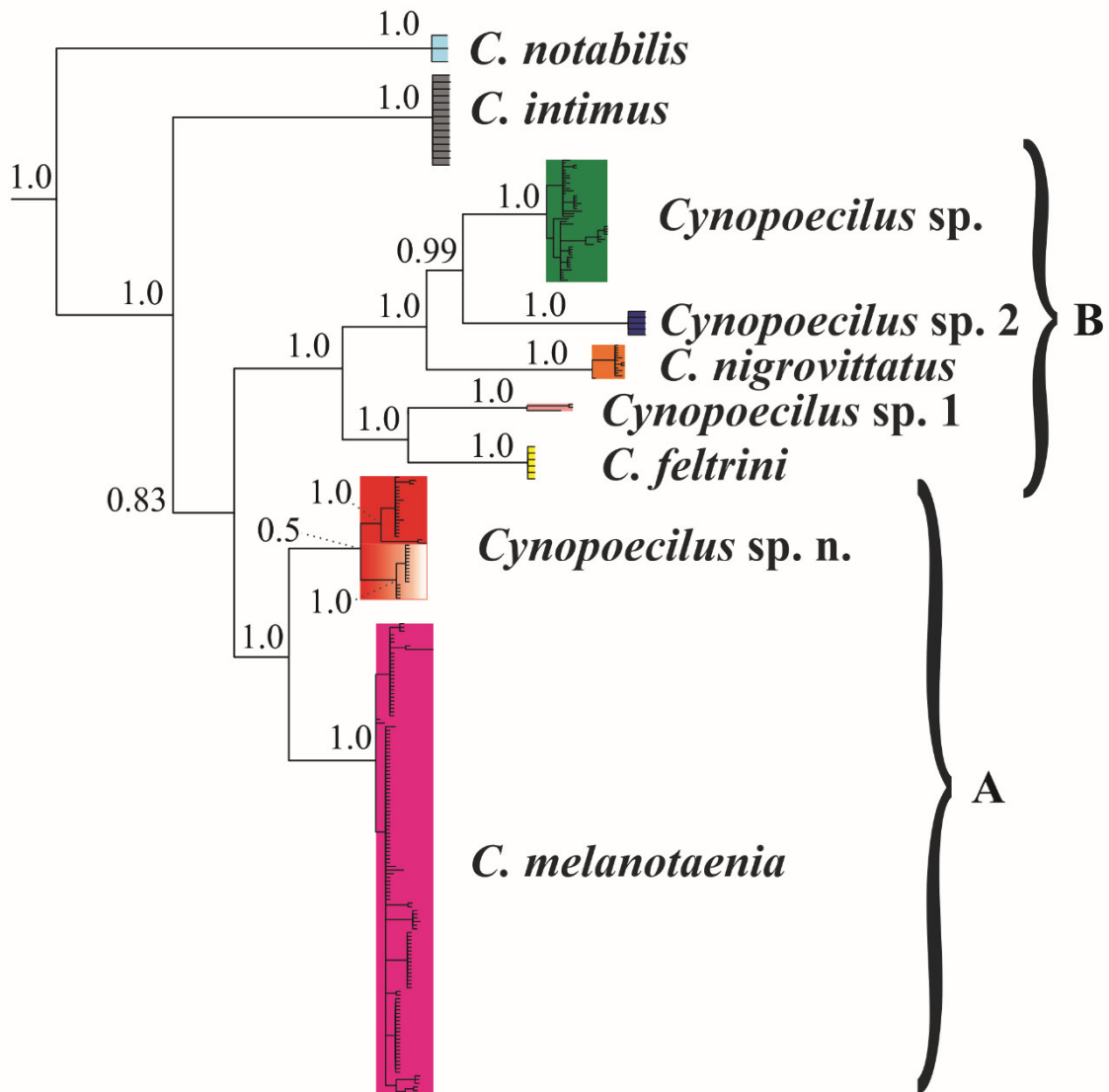
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735 **Figure 1** Bayesian Inference tree obtained from the concatenated dataset COI + RAG1
 736 emphasizing the subdivision of *Cynopoecilus* in ten clusters, among which two
 737 encompass each of the different populations of *Cynopoecilus* sp. n. Numbers above
 738 branches refer to the Bayesian posterior probability of each clade. Colours are in
 739 accordance with distribution patterns presented in Figure 5.

740



741

742 **Figure 2** Males (A and B) and female (C) paratypes of *Cynopoecilus* sp. n. from
 743 Eldorado do Sul municipality, type locality, Rio Grande do Sul, southern Brazil. Photos
 744 by Matheus Volcan.



745

746 **Figure 3** Males paratypes of *Cynopoecilus* sp. n. from Arroio dos Ratos municipality,
 747 Rio Grande do Sul, southern Brazil. Photos by Matheus Volcan.



748

749 **Figure 4** Females paratypes of *Cynopoecilus* sp. n. from Arroio dos Ratos municipality,
 750 Rio Grande do Sul, southern Brazil. Photos by Matheus Volcan.

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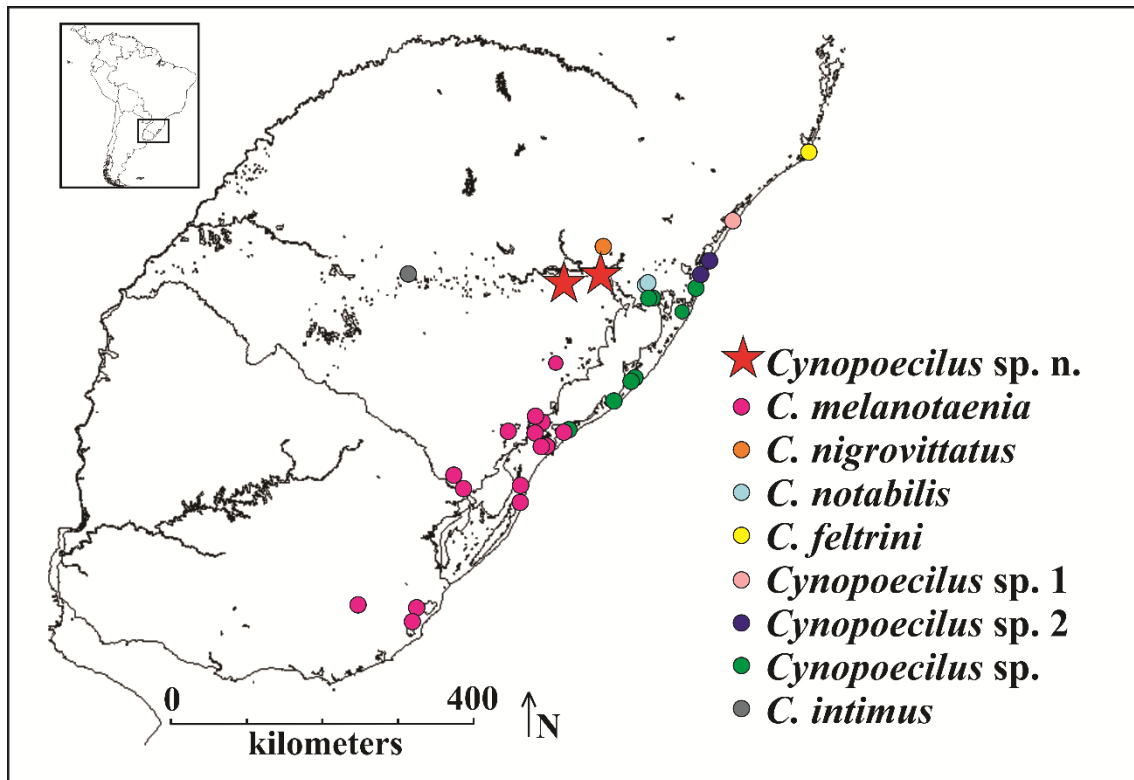


Figure 5 Geographic distribution of *Cynopoecilus* lineages, with an emphasis to the distribution of the two populations of *Cynopoecilus* sp. n. Source: Costa (2002), Costa *et al.* (2016), Ferrer *et al.* (2014) and Garcez *et al.* (2020).



Figure 6 Type locality of *Cynopoecilus* sp. n., a temporary pool in the floodplain of Jacuí River, Eldorado do Sul, Brazil. Photo by Daiana Garcez.

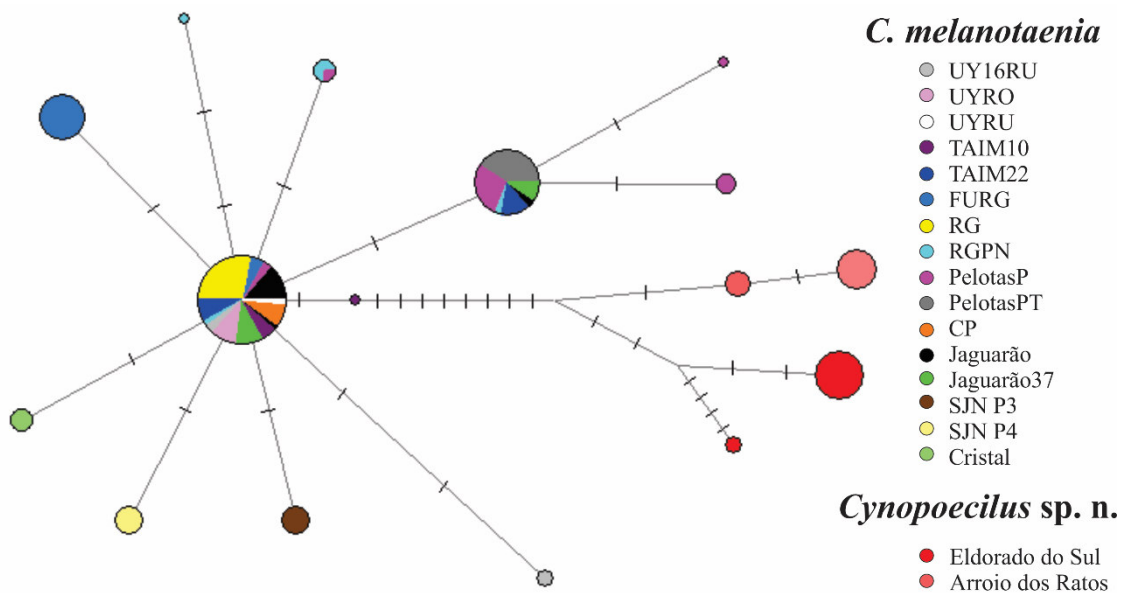
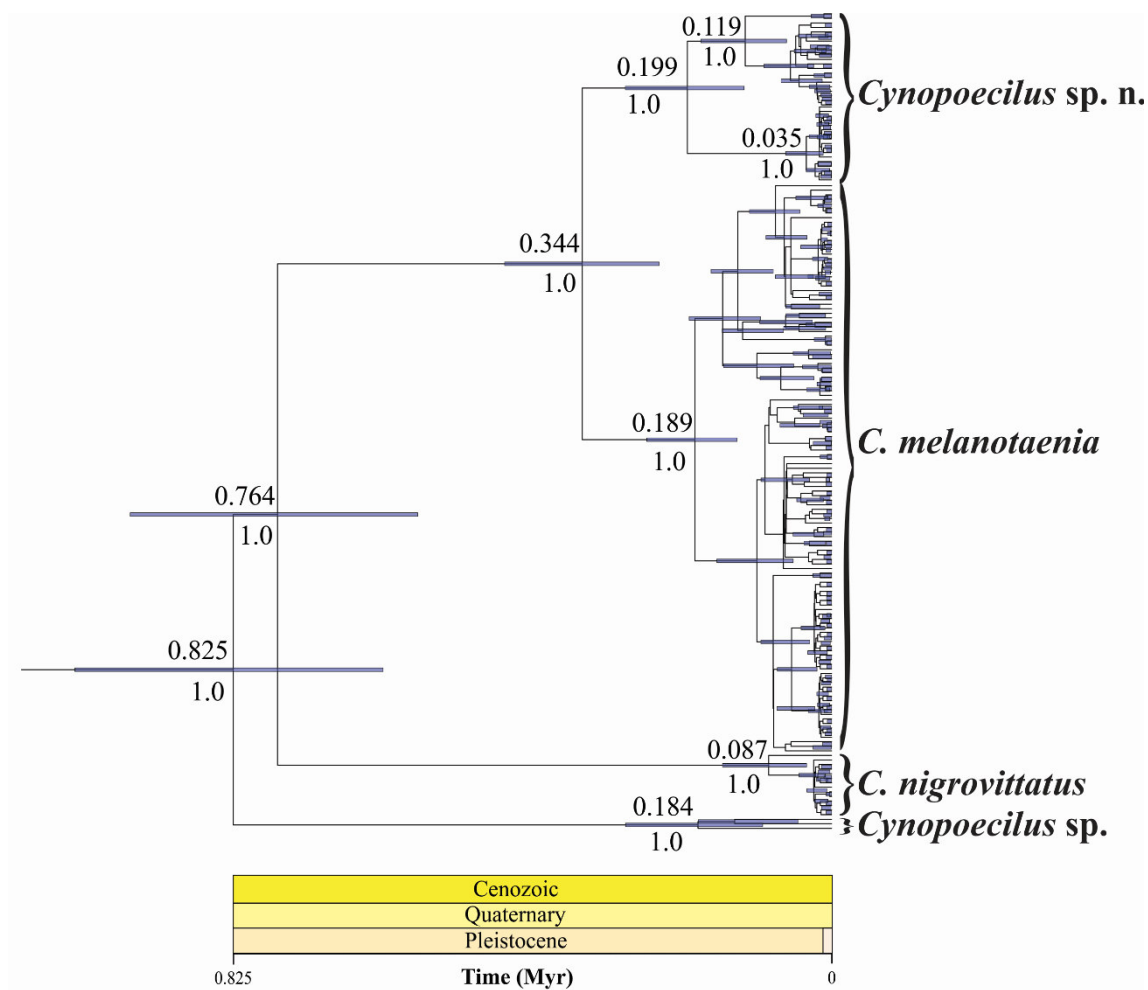


Figure 7 Haplotype network constructed with COI sequences of different populations of *C. melanotaenia* and *Cynopoecilus* sp. n. The dashes represent the number of mutational steps. The size of each circle is proportional to haplotype frequencies.



769

770 **Figure 8** Bayesian chronophylogenetic tree based on COI, ENC1 and RAG1 sequences
771 sampled for two different populations of *Cynopoecilus sp. n.*, *C. melanotaenia*, *C.*
772 *nigrovittatus* and *Cynopoecilus sp.* Values above nodes indicate divergence time
773 estimates. Values below internal branches represent support values, given by posterior
774 probabilities values in Bayesian analysis. Horizontal blue bars represent the 95% HPD
775 values for the estimated dates. This tree was rooted with sequences of *A. wolterstorffi*
776 sampled in both, the east and west margins of the Patos Lagoon, which were also
777 employed to calibrate the molecular clock rates (Garcez *et al.*, 2018).

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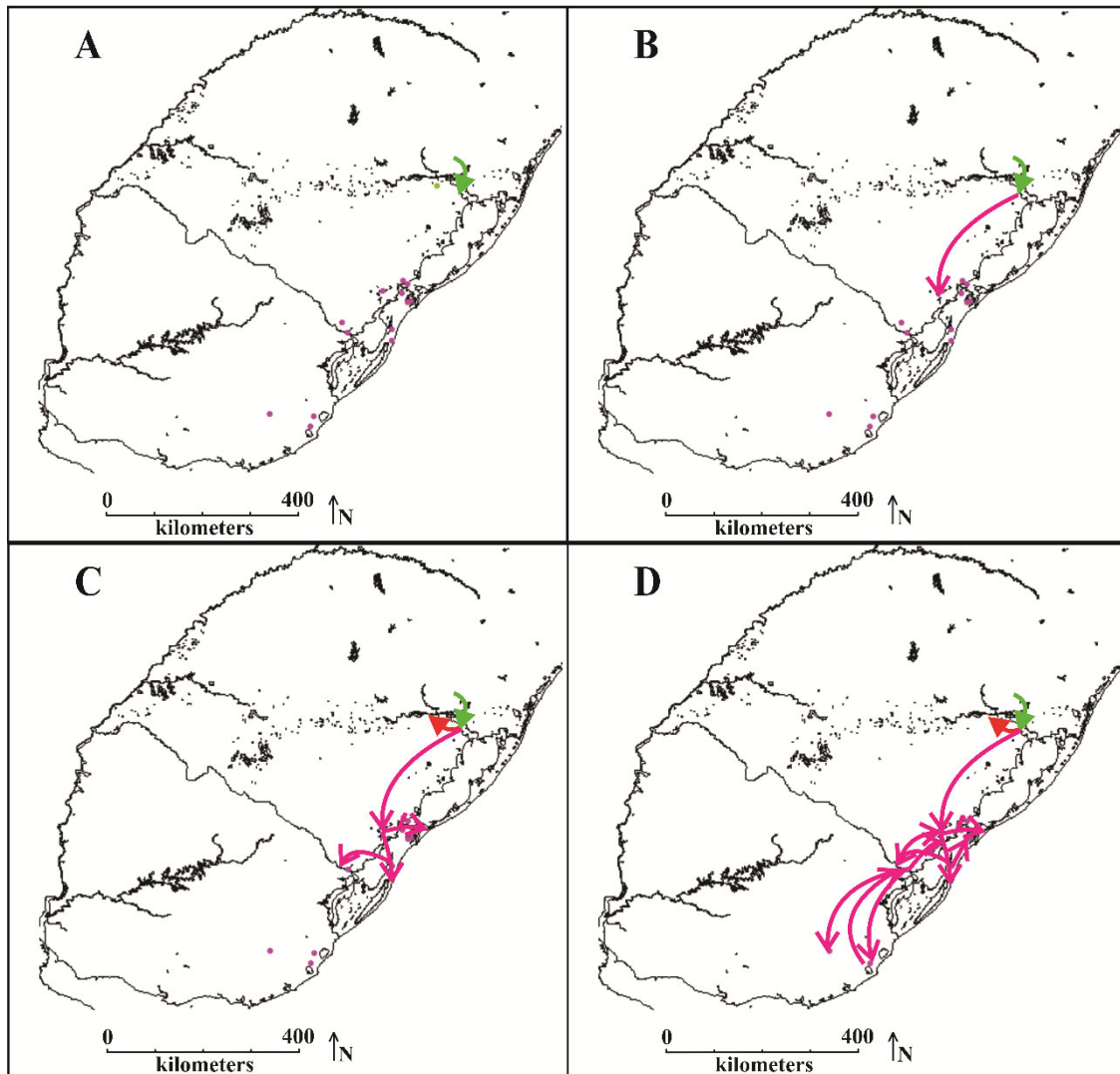


Figure 9 Spatial diffusion over time showing a north-south colonization route, through different time spans: (A) 150 Kya, (B) 75 Kya, (C) 33 Kya, (D) present. Green: colonization route between *C. nigrovittatus* and *Cynopoecilus* sp. n.; red: colonization route between the two populations of *Cynopoecilus* sp. n.; pink: colonization route between *Cynopoecilus* sp. n. and *C. melanotaenia* and further dispersion of the later. Figure generated on Google Earth through SPREAD analysis.

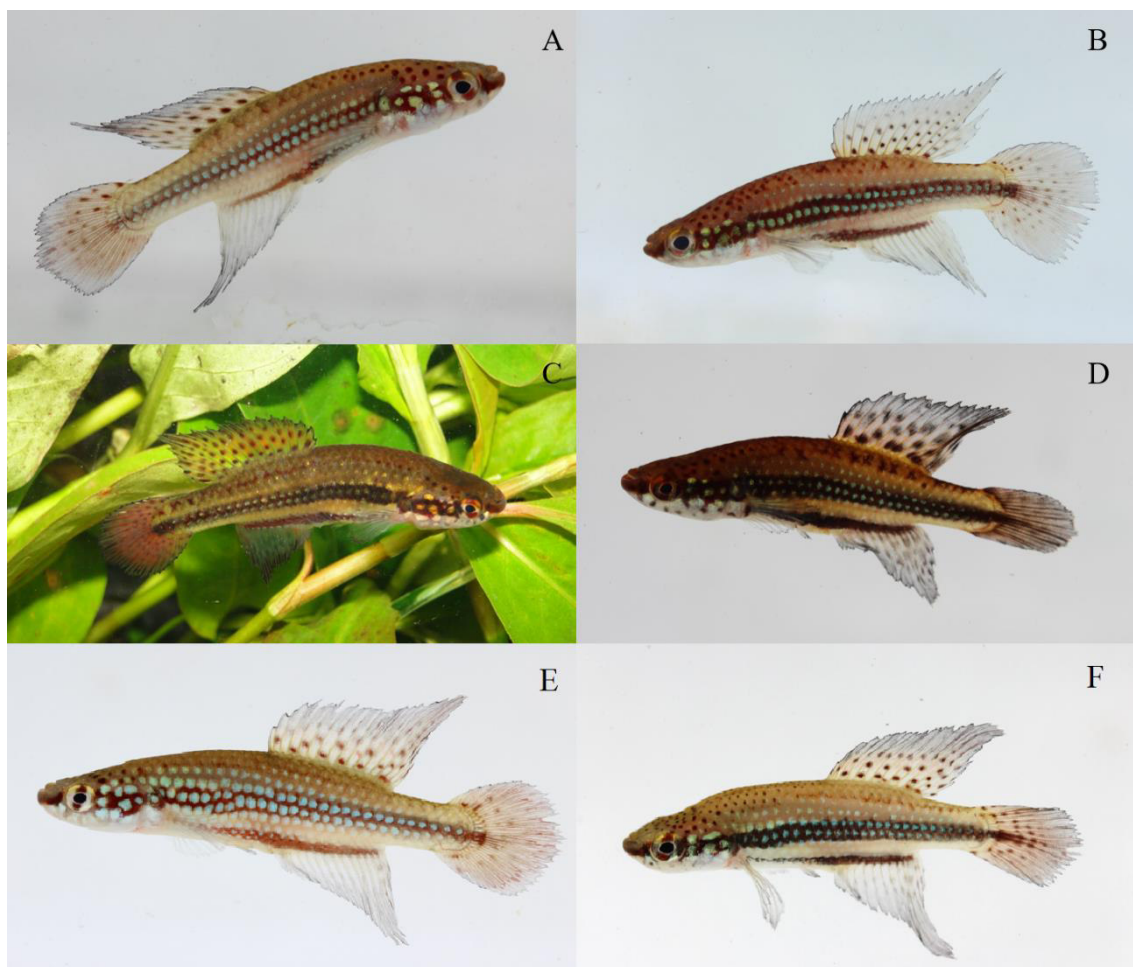


Figure 10 Males of representative specimens of *Cynopoecilus melanotaenia* complex recorded in Brazil (not preserved). (A) *C. feltrini*; (B) *C. nigrovittatus*; (C) *C. melanotaenia*; (D) *C. intimus*; (E and F) *Cynopoecilus* sp. n. Photos by Matheus Volcan.



Figure 11 Females of representative specimens of *Cynopoecilus melanotaenia* complex recorded in Brazil (not preserved). (A) *C. feltrini*; (B) *C. nigrovittatus*; (C) *C. melanotaenia*; (D) *C. intimus*; (E and F) *Cynopoecilus* sp. n. Photos by Matheus Volcan.

SUPPORTING INFORMATION

Table S1 List of sampling localities for specimens used in molecular analysis, with their respective geographical coordinates and GenBank accession numbers.

Supporting Information

Colonization history of *Cynopoecilus* (Cyprinodontiformes: Rivulidae) across the western margin of the Patos-Mirim Lagoon System, with the description of a new species

D. K. Garcez, M. V. Volcan and L. J. Robe

Table S1 List of sampling localities for specimens used in molecular analysis, with their respective geographical coordinates and GenBank accession numbers.

Table S1 List of sampling localities for specimens used in molecular analysis, with their respective geographical coordinates and GenBank accession numbers

N	Identification code	Species	Population	Coordinates	GenBank (COI)	GenBank (RAG1)	GenBank (ENC1)	Voucher
1	16RU1	<i>C. melanotaenia</i>	Uruguay Ruta 16	-34.031301 -53.823860	MK836470	MK836730		
2	16RU2	<i>C. melanotaenia</i>	Uruguay Ruta 16	-34.031301 -53.823860	MK836471	MK836731		
3	16RU3	<i>C. melanotaenia</i>	Uruguay Ruta 16	-34.031301 -53.823860	MK836472	MK836732		
4	16RU4	<i>C. melanotaenia</i>	Uruguay Ruta 16	-34.031301 -53.823860	MK836473	MK836733		
5	RO1	<i>C. melanotaenia</i>	Uruguay Laguna Negra	-34.005554 -53.585891	MK836677	MK836937		
6	RO3	<i>C. melanotaenia</i>	Uruguay Laguna Negra	-34.005554 -53.585891	MK836678	MK836938		
7	RO4	<i>C. melanotaenia</i>	Uruguay Laguna Negra	-34.005554 -53.585891	MK836679	MK836939		
8	RO5	<i>C. melanotaenia</i>	Uruguay Laguna Negra	-34.005554 -53.585891	MK836680	MK836940		
9	RO6	<i>C. melanotaenia</i>	Uruguay Laguna Negra	-34.005554 -53.585891	MK836681	MK836941		
10	RO7	<i>C. melanotaenia</i>	Uruguay Laguna Negra	-34.005554 -53.585891	MK836682	MK836942		
11	RU9	<i>C. melanotaenia</i>	Uruguay Ruta 9	-34.213551 -53.883296	MK836683	MK836943		
12	10T1	<i>C. melanotaenia</i>	Rio Grande – TAIM10	-32.554250 -52.495500	MK836466	MK836726	MW737028	MCP* 54235
13	10T2	<i>C. melanotaenia</i>	Rio Grande – TAIM10	-32.554250 -52.495500	MK836467	MK836727	MW737029	MCP 54235
14	10T3	<i>C. melanotaenia</i>	Rio Grande – TAIM10	-32.554250 -52.495500	MK836468	MK836728	MW737030	MCP 54235
15	10T4	<i>C. melanotaenia</i>	Rio Grande – TAIM10	-32.554250 -52.495500	MK836469	MK836729	MW737031	MCP 54235
16	22T1	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836474	MK836734	MW737032	MCP 54235
17	22T10	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836475	MK836735		MCP 54235
18	22T2	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836476	MK836736	MW737033	MCP 54235
19	22T3	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836477	MK836737	MW737034	MCP 54235
20	22T4	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MN326791	MK836738	MW737035	MCP 54235
21	22T5	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836478	MK836739	MW737036	
22	22T6	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836479	MK836740	MW737037	
23	22T7	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836480	MK836741	MW737038	
24	22T8	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836481	MK836742	MW737039	
25	22T9	<i>C. melanotaenia</i>	Rio Grande – TAIM22	-32.748000 -52.494972	MK836482		MW737040	

26	CP1	<i>C. melanotaenia</i>	Capão do Leão	-31.892497 -52.656845	MK836510	MK836770	MW737041	MCP 54236
27	CP2	<i>C. melanotaenia</i>	Capão do Leão	-31.892497 -52.656845	MK836511	MK836771	MW737042	MCP 54236
28	CP3	<i>C. melanotaenia</i>	Capão do Leão	-31.892497 -52.656845	MK836512	MK836772		MCP 54236
29	CP4	<i>C. melanotaenia</i>	Capão do Leão	-31.892497 -52.656845	MK836513	MK836773	MW737043	MCP 54236
30	CP5	<i>C. melanotaenia</i>	Capão do Leão	-31.892497 -52.656845	MK836514	MK836774	MW737044	
31	CP6	<i>C. melanotaenia</i>	Capão do Leão	-31.892497 -52.656845		MK836996	MW737045	
32	F1	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836515	MK836775	MW737046	
33	F10	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836516	MK836776	MW737053	
34	F11	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836517	MK836777	MW737054	
35	F12	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836518	MK836778		MCP 54237
36	F13	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836519	MK836779	MW737055	MCP 54237
37	F14	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836520	MK836780	MW737056	MCP 54237
38	F15	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836521	MK836781	MW737057	MCP 54237
39	F16	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836522	MK836782	MW737058	
40	F17	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836523	MK836783	MW737059	
41	F18	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836524	MK836784	MW737060	
42	F19	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836525	MK836785	MW737061	
43	F2	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836526	MK836786	MW737047	
44	F20	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836527	MK836787	MW737062	
45	F21	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836528	MK836788	MW737063	
46	F3	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836529	MK836789	MW737048	
47	F4	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836530	MK836790	MW737049	
48	F5	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836531	MK836791	MW737050	
49	F6	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836532	MK836792	MW737051	
50	F8	<i>C. melanotaenia</i>	Rio Grande – FURG	-32.070227 -52.160778	MK836533	MK836793	MW737052	
51	R1	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836660	MK836920	MW737203	MCP 54238
52	R11	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836661	MK836921	MW737211	MCP 54238
53	R12	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836662	MK836922	MW737212	MCP 54238
54	R14	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836663	MK836923	MW737213	MCP 54238
55	R15	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836664	MK836924	MW737214	
56	R17	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836665	MK836925	MW737215	

57	R19	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836666	MK836926	MW737216	
58	R2	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836667	MK836927	MW737204	
59	R21	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836668	MK836928	MW737217	
60	R22	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836669	MK836929	MW737218	
61	R3	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836670	MK836930	MW737205	
62	R4	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836671	MK836931	MW737206	
63	R5	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836672	MK836932	MW737207	
64	R6	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836673	MK836933	MW737208	
65	R7	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836674	MK836934		
66	R8	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836675	MK836935	MW737209	
67	R9	<i>C. melanotaenia</i>	Rio Grande – RG	-32.088456 -52.225002	MK836676	MK836936	MW737210	
68	PN1	<i>C. melanotaenia</i>	Rio Grande – Povo Novo	-31.922222 -52.313611	MK836641	MK836901	MW737184	
69	PN11	<i>C. melanotaenia</i>	Rio Grande – Povo Novo	-31.922222 -52.313611	MK836642	MK836902	MW737189	
70	PN2	<i>C. melanotaenia</i>	Rio Grande – Povo Novo	-31.922222 -52.313611	MK836643	MK836903	MW737185	MCP 54239
71	PN3	<i>C. melanotaenia</i>	Rio Grande – Povo Novo	-31.922222 -52.313611	MK836644	MK836904	MW737186	MCP 54239
72	PN4	<i>C. melanotaenia</i>	Rio Grande – Povo Novo	-31.922222 -52.313611	MK836645	MK836905	MW737187	MCP 54239
73	PN5	<i>C. melanotaenia</i>	Rio Grande – Povo Novo	-31.922222 -52.313611	MK836646	MK836906	MW737188	MCP 54239
74	P11	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836611	MK836871	MW737160	
75	P12	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836612	MK836872	MW737161	
76	P13	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836613	MK836873	MW737162	
77	P14	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836614	MK836874	MW737163	MCP 54240
78	P15	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836615	MK836875	MW737164	MCP 54240
79	P16	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836616	MK836876	MW737165	MCP 54240
80	P18	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836617	MK836877	MW737166	MCP 54240
81	P2	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836618	MK836878	MW737153	
82	P20	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836619	MK836879	MW737167	
83	P3	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836620	MK836880	MW737154	
84	P4	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836621	MK836881	MW737155	
85	P5	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836638	MK836898	MW737156	
86	P6	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836639	MK836899	MW737157	
87	P7	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MK836640	MK836900	MW737158	

88	P8	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MN326793		MW737159	
89	P22	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411	MN326794		MW737168	
90	P23	<i>C. melanotaenia</i>	Pelotas – Pontal	-31.778426 -52.231411		MK836999		
91	PT1	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836647	MK836907	MW737190	MCP 54241
92	PT10	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836648	MK836908	MW737197	MCP 54241
93	PT11	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836649	MK836909	MW737198	MCP 54241
94	PT12	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836650	MK836910	MW737199	MCP 54241
95	PT14	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836651	MK836911	MW737200	
96	PT15	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836652	MK836912	MW737201	
97	PT16	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836653	MK836913	MW737202	
98	PT2	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836654	MK836914	MW737191	
99	PT3	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836655	MK836915	MW737192	
100	PT4	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836656	MK836916	MW737193	
101	PT5	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836657	MK836917	MW737194	
102	PT6	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836658	MK836918	MW737195	
103	PT7	<i>C. melanotaenia</i>	Pelotas – Pelotapes	-31.713333 -52.300833	MK836659	MK836919	MW737196	
104	J11	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836536	MK836796	MW737071	MCP 54242
105	J12	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836537	MK836797	MW737072	MCP 54242
106	J14	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836538	MK836798	MW737073	MCP 54242
107	J16	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836539	MK836799	MW737074	MCP 54242
108	J2	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836540	MK836800	MW737065	
109	J3	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836541	MK836801	MW737066	
110	J4	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836542	MK836802	MW737067	
111	J5	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836543	MK836803	MW737068	
112	J6	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836544	MK836804	MW737069	
113	J7	<i>C. melanotaenia</i>	Jaguarão	-32.438333 -53.340556	MK836545	MK836805	MW737070	
114	37A	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836485	MK836745	MW737075	UFRGS 18072
115	37B	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836486	MK836746	MW737076	UFRGS 18072
116	37C	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836487	MK836747	MW737077	UFRGS 18072
117	37D	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836488	MK836748	MW737078	UFRGS 18072
118	37E	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836489	MK836749	MW737079	UFRGS 18072

119	37F	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836490	MK836750	MW737080	UFRGS 18072
120	37G	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836491	MK836751	MW737081	UFRGS 18072
121	37H	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836492	MK836752	MW737082	UFRGS 18072
122	37I	<i>C. melanotaenia</i>	Jaguarão – 37	-32.598778 -53.241444	MK836493	MK836753	MW737083	UFRGS 18072
123	Cri1	<i>C. melanotaenia</i>	Cristal	-31.078056 -52.038333	KT823671			
124	Cri2	<i>C. melanotaenia</i>	Cristal	-31.078056 -52.038333	KT823672			
125	Cri3	<i>C. melanotaenia</i>	Cristal	-31.078056 -52.038333	KT823673			
126	Cri4	<i>C. melanotaenia</i>	Cristal	-31.078056 -52.038333	KT823674			
127	N13	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050	MK836554	MK836814	MW737095	MCP 54243
128	N14	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050	MK836555	MK836815	MW737096	MCP 54243
129	N3	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050	MK836556	MK836816	MW737092	MCP 54243
130	N4	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050	MK836557	MK836817	MW737093	
131	N6	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050	MK836558	MK836818	MW737094	MCP 54243
132	N2	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050		MK836997		
133	N5	<i>C. melanotaenia</i>	São José do Norte – 3	-31.917200 -51.943050		MK836998		
134	SM1	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836690	MK836950	MW737219	MCP 54244
135	SM10	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836691	MK836951	MW737228	MCP 54244
136	SM11	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836692	MK836952	MW737229	MCP 54244
137	SM12	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836693	MK836953	MW737230	MCP 54244
138	SM13	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836694	MK836954	MW737231	
139	SM14	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836695	MK836955	MW737232	
140	SM2	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836696	MK836956	MW737220	
141	SM3	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836697	MK836957	MW737221	
142	SM4	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836698	MK836958	MW737222	
143	SM5	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836699	MK836959	MW737223	
144	SM6	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836700	MK836960	MW737224	
145	SM7	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836701	MK836961	MW737225	
146	SM8	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836702	MK836962	MW737226	
147	SM9	<i>C. intimus</i>	Santa Maria	-29.993861 -53.936944	MK836703	MK836963	MW737227	
148	NE1	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836576	MK836836	MW737114	MCP 54245
149	NE10	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836577	MK836837	MW737122	MCP 54245

150	NE11	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836578	MK836838	MW737123	MCP 54245
151	NE12	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836579	MK836839	MW737124	MCP 54245
152	NE13	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836580	MK836840	MW737125	
153	NE14	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836581	MK836841	MW737126	
154	NE15	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836582	MK836842	MW737127	
155	NE17	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836583	MK836843	MW737128	
156	NE18	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836584	MK836844	MW737129	
157	NE19	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836585	MK836845	MW737130	
158	NE2	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836586	MK836846	MW737115	
159	NE20	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836587	MK836847	MW737131	
160	NE21	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836588	MK836848	MW737132	
161	NE22	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836589	MK836849	MW737133	
162	NE23	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836590	MK836850	MW737134	
163	NE3	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836591	MK836851	MW737116	
164	NE5	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836592	MK836852	MW737117	
165	NE6	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836593	MK836853	MW737118	
166	NE7	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836594	MK836854	MW737119	
167	NE8	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836595	MK836855	MW737120	
168	NE9	<i>Cynopoecilus</i> sp. n.	Eldorado do Sul	-30.003054 -51.269481	MK836596	MK836856	MW737121	
169	NA1	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836559	MK836819	MW737097	
170	NA11	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836560	MK836820	MW737106	
171	NA12	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836561	MK836821	MW737107	MCP 54246
172	NA13	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836562	MK836822	MW737108	MCP 54246
173	NA14	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836563	MK836823	MW737109	MCP 54246
174	NA15	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836564	MK836824	MW737110	MCP 54246
175	NA17	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836565	MK836825	MW737111	
176	NA19	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836566	MK836826	MW737112	
177	NA2	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836567	MK836827	MW737098	
178	NA20	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836568	MK836828	MW737113	
179	NA3	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836569	MK836829	MW737099	
180	NA4	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836570	MK836830	MW737100	

181	NA5	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836571	MK836831	MW737101	
182	NA6	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836572	MK836832	MW737102	
183	NA7	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836573	MK836833	MW737103	
184	NA8	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836574	MK836834	MW737104	
185	NA9	<i>Cynopoecilus</i> sp. n.	Arroio dos Ratos	-30.104485 -51.722630	MK836575	MK836835	MW737105	
186	NM1	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836597	MK836857	MW737135	MCP 54247
187	NM10	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836598	MK836858	MW737144	MCP 54247
188	NM11	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836599	MK836859	MW737145	MCP 54247
189	NM12	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836600	MK836860	MW737146	MCP 54247
190	NM13	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836601	MK836861	MW737147	
191	NM14	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836602	MK836862	MW737148	
192	NM15	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836603	MK836863	MW737149	
193	NM2	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836604	MK836864	MW737136	
194	NM4	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836605	MK836865	MW737138	
195	NM5	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836606	MK836866	MW737139	
196	NM6	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836607	MK836867	MW737140	
197	NM7	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836608	MK836868	MW737141	
198	NM8	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836609	MK836869	MW737142	
199	NM9	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MK836610	MK836870	MW737143	
200	NM3	<i>C. nigrovittatus</i>	Montenegro	-29.668598 -51.429879	MN326792		MW737137	
201	27B	<i>C. notabilis</i>	Águas claras	-30.095694 -50.849722	MK836483	MK836743	MW737150	UFRGS 16439
202	27C	<i>C. notabilis</i>	Águas claras	-30.095694 -50.849722	MK836484	MK836744	MW737151	UFRGS 16439
203	27A	<i>C. notabilis</i>	Águas claras	-30.095694 -50.849722		MK836986		UFRGS 16439
204	27D	<i>C. notabilis</i>	Águas claras	-30.095694 -50.849722		MK836987		UFRGS 16439
205	52C	<i>C. notabilis</i>	Águas claras	-30.132500 -50.889444	MK836494	MK836754	MW737152	UFRGS 19629
206	52D	<i>C. notabilis</i>	Águas claras	-30.132500 -50.889444		MK836988		UFRGS 19629
207	52E	<i>C. notabilis</i>	Águas claras	-30.132500 -50.889444		MK836989		UFRGS 19629
208	52F	<i>C. notabilis</i>	Águas claras	-30.132500 -50.889444		MK836990		UFRGS 19629
209	UFRJ	<i>C. notabilis</i>	Águas claras	-30.096667 -50.851667	KT590068			
210	SC10	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722	MK836684	MK836944		
211	SC2	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722	MK836685	MK836945		

212	SC3	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722	MK836686	MK836946		
213	SC4	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722	MK836687	MK836947		
214	SC6	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722	MK836688	MK836948		
215	SC9	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722	MK836689	MK836949		
216	SC1	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722		MK837000		
217	SC5	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722		MK837001		
218	SC7	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722		MK837002		
219	SC8	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722		MK837003		
220	SC11	<i>C. feltrini</i>	Laguna – SC	-28.511670 -48.799722		MK837004		
221	MT1	<i>Cynopoecilus</i> sp. 1	Torres	-29.337806 -49.751972	MK836546	MK836806	MW737084	MCP 54248
222	MT2	<i>Cynopoecilus</i> sp. 1	Torres	-29.337806 -49.751972	MK836547	MK836807	MW737085	MCP 54248
223	MT3	<i>Cynopoecilus</i> sp. 1	Torres	-29.337806 -49.751972	MK836548	MK836808	MW737086	MCP 54248
224	MX1	<i>Cynopoecilus</i> sp. 2	Xangri-lá	-29.821833 -50.066389	MK836549	MK836809	MW737087	MCP 54249
225	MX2	<i>Cynopoecilus</i> sp. 2	Xangri-lá	-29.821833 -50.066389	MK836550	MK836810	MW737088	MCP 54249
226	MX3	<i>Cynopoecilus</i> sp. 2	Xangri-lá	-29.821833 -50.066389	MK836551	MK836811	MW737089	MCP 54249
227	MX4	<i>Cynopoecilus</i> sp. 2	Xangri-lá	-29.821833 -50.066389	MK836552	MK836812	MW737090	MCP 54249
228	MX5	<i>Cynopoecilus</i> sp. 2	Xangri-lá	-29.821833 -50.066389	MK836553	MK836813	MW737091	
229	O3	<i>Cynopoecilus</i> sp. 2	Osório	-29.988889 -50.192222	KT823653			
230	O4	<i>Cynopoecilus</i> sp. 2	Osório	-29.988889 -50.192222	KT823654			
231	O5	<i>Cynopoecilus</i> sp. 2	Osório	-29.988889 -50.192222	KT823655			
232	Ci6	<i>Cynopoecilus</i> sp.	Cidreira	-30.152500 -50.240278	KT823656			
233	Ci7	<i>Cynopoecilus</i> sp.	Cidreira	-30.152500 -50.240278	KT823657			
234	Ci8	<i>Cynopoecilus</i> sp.	Cidreira	-30.152500 -50.240278	KT823658			
235	78A	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836495	MK836755	MW737233	UFRGS 20689
236	78B	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836496	MK836756	MW737234	UFRGS 20689
237	78C	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836497	MK836757	MW737235	UFRGS 20689
238	78D	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836498	MK836758	MW737236	UFRGS 20689
239	78G	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836499	MK836759	MW737237	UFRGS 20689
240	78J	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836500	MK836760	MW737238	UFRGS 20689
241	78N	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111	MK836501	MK836761	MW737239	UFRGS 20689
242	78F	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111		MK836991		UFRGS 20689

243	78I	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111		MK836992		UFRGS 20689
244	78O	<i>Cynopoecilus</i> sp.	Viamão – 78	-30.286944 -50.791111		MK836993		UFRGS 20689
245	81B	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836502	MK836762	MW737240	UFRGS 20693
246	81C	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836503	MK836763	MW737241	UFRGS 20693
247	81D	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836504	MK836764	MW737242	UFRGS 20693
248	81E	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836505	MK836765	MW737243	UFRGS 20693
249	81G	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836506	MK836766	MW737244	UFRGS 20693
250	81J	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836507	MK836767	MW737245	UFRGS 20693
251	81K	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836508	MK836768	MW737246	UFRGS 20693
252	81N	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056	MK836509	MK836769	MW737247	UFRGS 20693
253	81F	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056		MK836994		UFRGS 20693
254	81L	<i>Cynopoecilus</i> sp.	Viamão – 81	-30.279167 -50.848056		MK836995		UFRGS 20693
255	SP1	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836704	MK836964	MW737248	MCP 54250
256	SP2	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836705	MK836965	MW737249	MCP 54250
257	SP3	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836706	MK836966	MW737250	MCP 54250
258	SP4	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836707	MK836967	MW737251	MCP 54250
259	SP5	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836708	MK836968	MW737252	
260	SP6	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836709	MK836969		
261	SP7	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836710	MK836970	MW737253	
262	SP8	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836711	MK836971	MW737254	
263	SP9	<i>Cynopoecilus</i> sp.	Palmares do Sul	-30.451111 -50.431667	MK836712	MK836972	MW737255	
264	FTM8	<i>Cynopoecilus</i> sp.	Tavares – Talha Mar	-31.240750 -51.004880	MK836534	MK836794	MW737064	MCP 54251
265	FTM9	<i>Cynopoecilus</i> sp.	Tavares – Talha Mar	-31.240750 -51.004880	MK836535	MK836795		MCP 54251
266	T1	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836713	MK836973	MW737256	
267	T11	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836714	MK836974	MW737260	MCP 54252
268	T14	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836715	MK836975	MW737261	MCP 54252
269	T16	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836716	MK836976	MW737262	MCP 54252
270	T23	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836717	MK836977	MW737263	MCP 54252
271	T24	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836718	MK836978	MW737264	
272	T26	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836719	MK836979	MW737265	
273	T3	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836720	MK836980	MW737257	

274	T4	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836721	MK836981	MW737258	
275	T9	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523	MK836722	MK836982	MW737259	
276	T22	<i>Cynopoecilus</i> sp.	Tavares	-31.291687 -51.081523		MK837005		
277	X3	<i>Cynopoecilus</i> sp.	São José do Norte – BujX	-31.527200 -51.291000	MK836723	MK836983	MW737266	MCP 54253
278	X5	<i>Cynopoecilus</i> sp.	São José do Norte – BujX	-31.527200 -51.291000	MK836724	MK836984	MW737267	MCP 54253
279	X7	<i>Cynopoecilus</i> sp.	São José do Norte – BujX	-31.527200 -51.291000	MK836725	MK836985	MW737268	MCP 54253
280	P41	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836622	MK836882	MW737169	MCP 54254
281	P410	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836623	MK836883	MW737178	MCP 54254
282	P411	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836624	MK836884	MW737179	MCP 54254
283	P412	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836625	MK836885	MW737180	MCP 54254
284	P413	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836626	MK836886		
285	P414	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836627	MK836887	MW737181	
286	P415	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836628	MK836888	MW737182	
287	P416	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836629	MK836889	MW737183	
288	P42	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836630	MK836890	MW737170	
289	P43	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836631	MK836891	MW737171	
290	P44	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836632	MK836892	MW737172	
291	P45	<i>C. melanotaenia</i>	São José do Norte – 4	-31.880160 -51.862027	MK836633	MK836893	MW737173	
292	P46	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836634	MK836894	MW737174	
293	P47	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836635	MK836895	MW737175	
294	P48	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836636	MK836896	MW737176	
295	P49	<i>Cynopoecilus</i> sp.	São José do Norte – 4	-31.880160 -51.862027	MK836637	MK836897	MW737177	

* MCP = MCT-PUCRS

CAPÍTULO 3

Manuscrito submetido para a revista *Biodiversity and Conservation*

**Hotspots of annual fish along the Patos-Mirim Lagoon System and adjacent areas, with
an evaluation of conservation priority for species and localities**

Daiana Kaster Garcez, Crislaine Barbosa, Murilo de Oliveira Fernandes, Matheus Vieira
Volcan, Lizandra Jaqueline Robe

Hotspots of annual fish along the Patos-Mirim Lagoon System and adjacent areas located on Southern Brazil, with an evaluation of conservation priority for species and localities

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Abstract

Rivulidae represents one of the most endangered taxa in Brazil and encompasses species known as annual fish because they possess unique adaptations allowed their survival in seasonal pools. Several Brazilian species of annual fish inhabit the Patos-Mirim Lagoon System (PMLS), which is considered a hotspot of these species despite not having defined regions for conservation of annual fish. Therefore, the aim of this study is to assist in the delimitation of micro-hotspots of endemism, sympatry, and richness of annual fish along the PMLS, while assessing conservation priorities of evolutionary lineages and areas. For this purpose, distribution data were compiled for 42 species or evolutionary lineages that occur along 144 pools located at the PMLS for evaluate patterns of distribution of endemism, sympatry, and richness. We propose a new index that considers measures of genetic diversity, phylogenetic positioning, and number of populations within and outside conservation units, used for evaluating conservation priority for 29 of these lineages and 112 of these pools. Among the evaluated species and lineages, eight were detected as strictly endemic, and 55 of the evaluated pools showed records of sympatry. The patterns of lineage richness showed a

heterogeneous distribution of annual fish along the sampling area, with seven grids presenting records for four or more lineages. Interestingly, these are concentrated in four larger areas, located on the Southeast and on the Southwest margins of the Patos Lagoon and on the Southeast and Center-West Margins of the Mirim Lagoon, which present several pools whose conservation is urging.

Keywords: *Austrolebias*; biodiversity; *Cynopoecilus*; endemism; sympatry.

Declarations

Funding

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Conflicts of interest/Competing interests

No conflict of interest

Ethics approval

All collections referred in this study were authorized by the Ministério do Meio Ambiente (MMA), at the Biodiversity Authorization and Information System (SISBIO) (process number 55651-3). This study was also approved by the Animal Ethics Committee of the Universidade Federal do Rio Grande (process number 23116.008163 / 2015-23).

63

64 **Introduction**

65 Brazil is one of the 17 megadiverse countries on earth (Mittermeier et al. 1997), harboring
66 approximately 10% of the world's biological diversity (Lewinsohn and Prado 2005). Part of
67 this biodiversity is explained by the extent and heterogeneity of its habitats and ecosystems,
68 which are distributed along six different biomes (Amazon, Caatinga, Cerrado, Atlantic Forest,
69 Pampa, and Pantanal), two of which are considered hotspots for the conservation of
70 biodiversity (Myers et al. 2000). However, only about 10% of the approximately 1.8 million
71 species estimated for this area are known by science (Lewinsohn and Prado 2005). This
72 causes great concern in face of the current biodiversity crisis, which is imposing an
73 accelerated loss of species and even ecosystems. In fact, there is evidence that humans are
74 causing the sixth mass extinction, either through induction of habitat loss and fragmentation,
75 through the introduction of invasive species and promotion of pathogen dispersal or through
76 the induction of climate changes (Myers 1990; Leakey and Lewin 1992; Dirzo and Raven
77 2003).

78 According to the last International Union for Conservation of Nature's red list of
79 threatened species (IUCN 2020), more than 35,500 species are somehow endangered, of
80 which 1,520 occur in Brazil. Concerning animals, the last Brazilian red list indicates that
81 1,182 species occurring along the country are endangered (ICMBio 2018). Of these, 311
82 (23.3%) are continental species of Actinopterygii, and 125 encompass species of Rivulidae.
83 Therefore, this family sums up about 40% of the Brazilian freshwater fish that are
84 endangered, a number that rises to 60% when only its southernmost state (Rio Grande do Sul)
85 is considered (Rio Grande do Sul 2014). Consequently, Rivulidae stands out as the most

threatened group of freshwater fish in Brazil, especially in extreme south Brazil (ICMBio 2018; Rio Grande do Sul 2014).

Rivulidae comprises 468 species, one-third of which occur in Brazilian territory (Costa 2008; Fricke et al. 2021). Most members of this family are popularly known as annual fish or seasonal killifish, as they possess unique adaptations that enable their survival in seasonal pools. Their characteristic annual life cycle is associated to a rapid development and early sexual maturation. Moreover, their eggs are resistant to desiccation and remain buried in the substrate in diapause when the temporary pools dry out and the adults die (Berois et al. 2015). Therefore, annual fish usually comprise small and isolated populations, which are subject to reduced effective population sizes, inbreeding and low levels of gene flow. Taken together, these forces often reduce intrapopulation genetic diversity, increasing the risk of mutational meltdown and inbreeding depression (Frankham et al. 2004). However, at another level, these forces eventually result in differentiation between populations, which may culminate in allopatric speciation events.

The peculiar distribution of these species, which intrinsically constitute small and isolated populations, and the extensive loss and fragmentation of wetlands help to explain the strong extinction threats currently faced by annual fish. Indeed, the extent of pools supporting the diversity of Neotropical annual fish has been greatly reduced due to a series of impacts, usually related to the expansion of agricultural or urban activities, and various types of ventures as construction and duplication of roads (Fontana et al. 2003; Volcan et al. 2009; Lanés and Maltchik 2010; Volcan et al. 2010; Volcan and Lanés 2018). In face of these threats, the Brazilian Ministry of Environment proposed in 2013 the “National Action Plan for the Conservation of Endangered Rivulid Fish - PAN Rivulidae” (ICMBio 2013), with the

objective of conserving the species of rivulids and their habitats. In fact, as annual fish are important for the the understanding of processes and the evolutionary history of animal biodiversity in general and in the Neotropical region, and for ethical issues related to the loss and extinction of species by human activities, their conservation is fundamental for the preservation of these ecosystems. Furthermore, annual fish can be important flagship species for the conservation of temporary pools and adjacent areas and the studies by Lanés et al. (2016) and Lanés et al. (2018) emphasize the importance of these species being considered as flag species to support conservation strategies and environmental licensing.

The Patos-Mirim Lagoon System (PMLS) located in southern Brazil harbors one of the highest numbers of endemic species of annual fish in the subtropical region of the Neotropics (Costa 2002). Since this region encompasses a relatively restricted geographical area containing an extraordinarily large concentration of biodiversity and endemism (Allen 2008), it can be considered a center of diversity and endemism of annual fish. Recent studies with annual fish that inhabit this region have shown the presence of high levels of cryptic diversity, whose origin and diversification is potentially related to the occurrence of the Quaternary Laguna-Barrier depositional systems (Tomazelli and Villwock 2005) or even to the presence of large water bodies that act as barriers to gene flow (Garcez et al. 2018; Barbosa et al. 2020; Garcez et al. 2020; Fernandes et al. 2021). The interference of these processes has been demonstrated, for example, for species included in the PAN Rivulidae [*Austrolebias wolterstorffi* (Garcez et al. 2018); *A. nigrofasciatus* (Barbosa et al. 2020) and *A. minuano* (Fernandes et al. 2021)]; for the *A. adloffi* species group considered as a whole (Barbosa 2019), and even for the genus *Cynopoecilus* (Garcez et al. 2020).

The *in situ* conservation aims to conserve biodiversity where it naturally occurs (Prates

and Irving 2015) and comprises one of the main mechanisms for the conservation of biodiversity provided by the Convention on Biological Diversity (CBD), signed by Brazil in 1992. Nevertheless, in order to elect priority areas for conservation, it is essential to characterize the patterns of distribution of current and historical biodiversity. As funding and human resources are both lacking to carry out inventories on the distribution of several taxa, we reconstructed here a list of all species or lineages (sensu Loureiro et al. 2018 and Costa 2002) of annual fish belonging to different taxonomic units that are currently known to occur along the PMLS (*A. adloffii*, *A. alexandri*, *A. bellottii*, *A. elongatus*, *A. jaegari* + *A. gymnoventris* and *A. luteoflammulatus* + *A. quirogai* species groups of *Austrolebias* and to the *Cynopoecilus* genus). This list was composed with their respective distribution, in order to demarcate micro-hotspots of endemism, sympatry and richness along the studied area, while assessing conservation priorities of lineages and areas.

We also propose a new index in the work, which simultaneously considers the level of genetic diversity, the phylogenetic positioning and the number of populations inside and outside the UCs to measure the conservation priority presented by each taxon. This mapping is expected to provide subsidies for the creation and implementation of public policies aimed at establishing areas for *in situ* conservation of annual fish and their habitats.

Materials and methods

Studied species or lineages

Most registers of annual fish along the PMLS comprise *A. wolterstorffi*, the *A. adloffii* species group and the *Cynopoecilus* genus (Lanés et al. 2018), which were thus elected to perform

genetic evaluations. The set of evolutionary lineages disentangled for these three taxonomic units with molecular analysis will be herein referred as “restricted dataset”. This dataset encompasses 29 lineages (see below) that were collected across 112 ponds. In addition, nine species that frequently occur in sympatry with these were also employed to perform endemism, sympatry, and richness analysis. These species are: *A. camaquensis*, *A. juanlangi* and *A. litzi* (*A. alexandri* group), *A. prognathus* (*A. elongatus* group), *A. jaegari*, *A. aff. gymnoventris* (*A. jaegari* + *A. gymnoventris* group), *A. luteoflammulatus*, *A. quirogai* (*A. luteoflammulatus* + *A. quirogai* group) and *A. univentripinnis* (*A. bellottii* group). Other four species across the Rio Grande do Sul state were also included in these analyzes in order to consider all species already registered for the PMLS: *A. cyaneus* and *A. paucisquama* (*A. alexandri* group), *A. cheradophilus* (*A. elongatus* group) and *A. melanoorus* (*A. bellottii* group). This yielded a larger number of species and lineages, which will be herein referred as the “complete dataset”. This dataset encompasses 42 lineages, with registers across 144 ponds.

Study area

The study area covers the PMLS and some adjacent areas, all located in the state of Rio Grande do Sul (RS), Brazil. The diversity of annual fish along 144 temporary pools distributed over 45 municipalities was evaluated through new collections, through compilations from the bibliography (Barbosa 2019; Barbosa et al. 2020; Costa 2006; Costa et al. 2016; Costa et al. 2017; Fernandes et al. 2021; Garcez et al. 2018; Garcez et al. 2020; García 2006; Lanés et al. 2013, 2014; Volcan et al. 2010, 2011a, 2011b, 2014, 2015, 2021), or from registers of the Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do

178 Rio Grande do Sul, Porto Alegre (MCP) and the Departamento de Zoologia, Universidade
179 Federal do Rio Grande do Sul, Porto Alegre (UFRGS), both, for the restricted and complete
180 set of species (Fig. 1, Supplementary Material – Table S1).

181 Of the 45 municipalities, 17 municipalities (Balneário Pinhal, Chuí, Cidreira, Gravataí,
182 Mostardas, Osório, Palmares do Sul, Porto Alegre, Rio Grande, Santa Vitória do Palmar,
183 Santo Antônio da Patrulha, São José do Norte, Tavares, Torres, Turuçu, Viamão and Xangri-
184 lá), as well as some sampling localities in Pelotas, are distributed along Quaternary deposits
185 of the Coastal Plain of RS (CPRS) (yellow in Fig. 1). This area was intensively modified
186 during the marine regressions and transgressions that occurred during the Pleistocene and
187 Holocene, which constituted a system known as the Laguna-Barrier System between 400,000
188 and 5,000 years ago (Tomazelli and Villwock 2005). This system lead to the formation of the
189 Patos, Mirim and Manguera Lagoons (Villwock and Tomazelli 2007).

190 Among the other municipalities, Arroio Grande, Cachoeirinha, Capão do Leão, Cristal,
191 Eldorado do Sul, Guaíba, Jaguarão, Pedro Osório, São Lourenço do Sul and the other
192 locations in Pelotas are part of the Alluvial Fan System of the CPRS, with deposits
193 accumulated in the Tertiary and reworked in the Quaternary in its most distal portions
194 (Villwock and Tomazelli 2007) (in blue in Fig. 1). Another 12 municipalities (Aceguá, Bagé,
195 Candiota, Canoas, Charqueadas, Hulha Negra, Montenegro, Pedras Altas, Rio Pardo, Santa
196 Maria, São Gabriel, and São Sepé) are located in the Central Depression, a region that
197 contains sedimentary and volcanic deposits from the Permian-Triassic, which are part of the
198 Paraná Basin (Villwock and Tomazelli 2007) (in cyan in Fig. 1). Lastly, Arroio dos Ratos,
199 Caçapava do Sul, Canguçu, Encruzilhada do Sul, Herval and Minas do Leão are located in the
200 Precambrian Shield or Crystalline Shield Sul-Rio-Grandense, an area that has igneous rocks

from the Precambrian period, greatly eroded by erosion (Villwock and Tomazelli 2007) (in pink in Fig. 1).

Collection and molecular procedures

New collections were performed in six temporary pools distributed over four municipalities, with the help of hand nets. The collected fishes were initially anesthetized with the use of eugenol 3,000 mg/ml, and then fixed in ethanol 95%. Specimens of *A. wolterstorffi*, of different species of the *A. adloffii* species group and of *Cynopoecilus* had their total DNA extracted from approximately 30 mg of the caudal peduncle. This DNA was then used in the amplification of mitochondrial (COI and/or Cyt *b*) and nuclear (ENC1) markers. COI was amplified using the primers LCO1480 and COX1R (5' GG YTCTTCRAARGTGTGATAS 3') (Costa and Amorim 2011) for *A. wolterstorffi* and the *A. adloffii* species group, and LCO1490 (5' GGTCAACAAATCATAAAGATATTGG 3') and COIb (5' CCAGAGAATAGAGGGAATCAGTG 3') (Folmer et al. 1994; Murphy et al. 1999, respectively) for *Cynopoecilus*. Primers L14735 (5' AAAAACCACCGTTGTTATTCAACTA 3') and CB3-H (5' GGCAAATAGGAARTATCATTC 3') (Palumbi et al. 1991; Wolf et al. 1999) were used in the amplification of Cyt *b*. For ENC1, primers F85 (5' GACATGCTGGAGTTTCAGGA 3') and R982 (5' ACTTGTTTRGCMACTGGGTCAAA 3') (Li et al. 2007) were employed for the different taxonomic units.

Phylogenetic analysis

Sequences previously characterized for *A. wolterstorffi* (Garcez et al. 2018), *A. nigrofasciatus* (Barbosa et al. 2020), *A. minuano*, *A. charrua*, *A. pongondo* (Fernandes et al. 2021), *A. adloffii* (Garcez et al. 2018; García et al. 2002; García 2006), *A. bagual*, *A. minuano* + *A. charrua*, *A. pelotapes*, *A. lourenciano* and *A. cheffei* (Volcan et al. 2021) and *Cynopoeilus* (Costa et al. 2016; Garcez et al. 2020) were downloaded from GenBank and added to the new set of sequences characterized here. Individual markers were aligned using the ClustalW algorithm, as performed by MEGA 7 (Kumar et al. 2016). After, a phylogenetic tree was reconstructed under Bayesian Analysis (BA) in Beast 1.10.4 (Suchard et al. 2018), using sequences of *Nothobranchius guentheri* (accession numbers MN099869 and KY031591 for COI and ENC1, respectively) and *Kryptolebias marmoratus* (accession numbers AF283503 and XM_017431259 for COI and ENC1, respectively) as outgroups. This analysis was performed with the simultaneous use of COI and ENC1 sequences, under unlinked nucleotide substitution and clock models, but linked gene trees. The nucleotide substitution models were selected based on the Akaike Information Criterion (AIC), as performed in MrModeltest2 (Nylander 2004). The BA encompassed 20,000,000 generations, with trees and parameters sampled every 2,000 iterations, and burn-in 10% of the initial results. Results were visualized in Tracer 1.7 (Rambaut et al. 2018) to evaluate convergence and stationarity and to ensure that all ESS (Effective Sample Sizes) were above 200. The maximum credibility tree was inferred by TreeAnnotator 1.5.4 (Drummond and Rambaut 2007) and edited in FigTree 1.4.3 (Rambaut and Drummond 2009). This tree was used to infer the evolutionary units of the restricted dataset, and to infer their respective phylogenetic position (see below).

245 Analysis of endemism, sympatry and richness distribution

246 Levels of endemism and sympatry, as well as richness distribution patterns were evaluated
247 from distribution data obtained for the complete dataset (Supplementary Material - Table S1).
248 Spatial richness distribution patterns were then obtained on Diva-GIS 7.5.0 (Hijmans et al.
249 2012). These analyses were performed considering the total number of different classes
250 (lineage richness) per cell, in grids ranging between longitudes -59 and -47, and latitudes -35
251 and -25, with cells of 0.25 x 0.25 (42 x 24 km = 1008 Km²) and a simple “point to grid”
252 procedure.

253

254 Conservation priority measures

255 Conservation priority of lineages were evaluated from distribution data, diversity and
256 phylogenetic estimates obtained for the restricted set of species. In this case, distribution data
257 was based on new collections as well as previous registers, whereas diversity and
258 phylogenetic estimates were based on the new information yielded by this study.
259 Conservation priority of temporary pools was evaluated for all the set of ponds harboring at
260 least one species of the restricted dataset.

261 Conservation priority of lineages was estimated based on: (a) the genetic diversity of
262 each lineage, as measured by the product between haplotypic (Hd) and nucleotide (π)
263 diversities estimated in DnaSP 5.10 (Librado and Rozas 2009) for COI and/or Cyt *b*, divided
264 by their respective substitution rates (μ) [(0.0274 and 0.0276, as estimated for *A. wolterstorffi*
265 by Garcez et al. (2018)]; (b) the phylogenetic position of each lineage (ph) on the topology of
266 the phylogenetic tree, following the method first described by Vane-Wright et al. (1991), in

which conservation priority is inferred from the number of internal nodes separating each lineage from the root of the tree; (c) the number of populations within Conservation Units (CUs) in each lineage ($\text{pops}_{\text{within}}$) and (d) the number of populations outside CUs in each lineage (pops_{out}). A value of 0.1 was added to all diversity values in (a) and the numbers of populations in (c) and (d), so none lineage retrieved zero values. The primary information value i thus estimated for each variable (a, b, c and d) was then used to establish the basic weight Q ($Q = \sum i/ij$, where j is each analyzed lineage) and W ($W = Qj/Q_{\text{min}}$) for each parameter in each lineage, following the methods employed by Costa (2012). The final index W_{lineage} was calculated as $W_{\text{lineage}} = (W_a + W_b) * (W_c * 0.5 + W_d)$. See that values of W are inversely proportional to the values of (a), (b), (c) and (d), so that higher values are recovered for lineages with low genetic diversity, with a basal phylogenetic positioning, and with low numbers of populations within and outside CUs, respectively. Since lineages with no population outside CUs are not currently good targets for *in situ* conservation, a W of 1 was assigned to variable (d) in these cases, as a way of avoiding an inflation of W_{lineage} .

Finally, the conservation priority of pools was estimated based on the sum of the values of W_{lineage} of all lineages it harbors. Moreover, the W_{pool} value thus obtained was multiplied by 0.5 if the pool is within a CUs and by 2 if it harbors strictly endemic species (considered here as a species that is registered from only one locality).

Results

In total, 623 and 370 sequences of COI and ENC1, respectively were included in the diversity and phylogenetic analysis (Supplementary Material – Table S2). Of these, 136 sequences of COI and 307 sequences of ENC1 are new for this study. The AIC test performed with

MrModeltest selected SYM + G substitution model for both matrixes. The phylogenetic evaluation allowed the definition of 28 reciprocally monophyletic lineages with occurrence in the PMLS (Fig. 2, Table 1). An additional subdivision was further considered for *A. nigrofasciatus*, based on evidence supporting the recent subdivision of the species in two different lineages that have not yet reached reciprocal monophyly (Barbosa et al. 2020). As discussed by Garcez et al. (2018 and 2020), Barbosa (2019) and Fernandes et al. (2021), most of these lineages shall represent distinct species, although some of them may still be considered incipient species.

Thus, the restricted dataset encompassed 29 lineages with occurrence along the PMLS: five for the *A. wolterstorffi* complex, 15 for the *A. adloffii* species group and nine for *Cynopoecilus*. Registers from all these species were added to those compiled for *A. camaquensis*, *A. cyaneus*, *A. juanlangi*, *A. litzi* and *A. paucisquama* (*A. alexandri* group), *A. cheradophilus* and *A. prognathus* (*A. elongatus* group), *A. jaegari*, *A. aff. gymnoventris* (*A. jaegari* + *A. gymnoventris* group), *A. luteoflammulatus*, *A. quirogai* (*A. luteoflammulatus* + *A. quirogai* group), *A. melanoorus* and *A. univentripinnis* (*A. bellottii* group) in order to constitute the complete dataset.

From the 42 lineages of annual fish encompassing the complete dataset, eight were revealed as strictly endemic, occurring on a single collection point. These are: *A. aff. nachtigalli*, *A. cheffei*, *A. arachan*, *A. jaegari*, *A. paucisquama*, *A. wolterstorffi_1*, *A. wolterstorffi_6* and *Cynopoecilus* sp. 1 (Fig. 3, Supplementary Material – Table S1). Also, 55 of the 144 pools presented evidence of sympatry among lineages (Supplementary Material – Table S1). In most of these cases, only two different lineages coexist in the same pool. However, in three sampling sites located in São José do Norte (pools 93, 95 and 98) and one

located in Chuí (pool 15), it was possible to simultaneously sample four distinct lineages (Supplementary Material – Table S1). The only lineages or species that were not found in sympatry with any of the others were: *A. arachan*, *A. cheradophilus*, *A. cyaneus*, *A. melanoorus*, *A. paucisquama*, *C. nigrovittatus*, *C. aff. nigrovittatus* 2, *C. notabilis*, *Cynopoecilus* sp. 1 and *Cynopoecilus* sp. 2. Species of the same group did not occur in sympatry, except for one point in Jaguarão where *A. aff. nachtigalli* co-occurred with *A. nachtigalli* (*A. adloffii* group) and three points in São José do Norte, registering the co-occurrence of *C. melanotaenia* and *Cynopoecilus* sp., *A. aff. minuano* 1 and *A. aff. minuano* 2 (*A. adloffii* group) and *A. wolterstorffi*_5 and 6 (*A. wolterstorffi* complex of the *A. elongatus* group).

Diversity estimates based on species richness corroborate the existence of a heterogeneous pattern of annual fish distribution across the PMLS and adjacent areas (Fig. 4). In this case, of the 56 grids sampled, 26 had records of a single lineage of annual fish, whereas only seven grids had records for four or more lineages (Fig. 4, Supplementary Material – Table S1). Four of these seven points are located near the margins of Patos Lagoon and have the following locations and compositions: point IX, located at the municipality of São José do Norte, on the Southeast margin of the Patos Lagoon, covers the area of pool 98, with records for *A. aff. minuano* 1, *A. aff. minuano* 2, *A. wolterstorffi*_5 and *Cynopoecilus* sp.; and point X, covering 11 pools located at the Southwest margin of the Patos Lagoon, along the municipalities of Capão do Leão, Pelotas and Rio Grande, presenting registers for *A. nigrofasciatus*_SG, *A. wolterstorffi*_2, *A. pongondo* and *C. melanotaenia*; point XIII, located at the municipality of São José do Norte, presenting four pools (among which pools 93, 95, 96 and 97) with records for *A. aff. minuano* 1, *A. wolterstorffi*_5, *A. wolterstorffi*_6, *C.*

melanotaenia, and *Cynopoecilus* sp.; point XIV, covering four pools located at the municipalities of São José do Norte, Pelotas and Rio Grande, presenting records for *A. nigrofasciatus*_SG, *A. pongondo*, *A. wolterstorffi*_2, *C. melanotaenia* and *Cynopoecilus* sp. (Fig. 4, Supplementary Material – Table S1). Conversely, the three other points with higher richness are located on the adjacencies of the Mirim Lagoon, both in the Center-West (points XI and XV) and the Southeast (point XII) margins of the Lagoon (Fig. 4, Supplementary Material – Table S1). Among these, point XV covers part of the municipality of Jaguarão, and presents registers for *A. aff. nactigalli* and *A. nactigalli*, occurring in sympatry at pool 31, *A. aff. reicherti* and *A. univentripinnis*, occurring in sympatry at pool 32, and *A. aff. reicherti* and *C. melanotaenia* occurring in sympatry at pool 33 (that is 6 km apart from the second). Besides these, point XI also occurs in the municipality of Jaguarão, and presents records for *A. aff. reicherti*, *A. cheradophilus*, *A. univentripinnis* and *C. melanotaenia*, and point XII occurs in the municipalities of Chuí and Santa Vitória do Palmar and presents records for *A. minuano* + *A. charrua*, *A. luteoflammulatus*, *A. prognathus* and *C. melanotaenia*.

Among the 29 lineages analyzed in the restricted dataset, *A. arachan*, *A. aff. nactigalli* and *A. wolterstorffi*_6 presented the highest values of W_{lineage} , and thus the highest conservation priorities (Table 2). In fact, *A. arachan* presented the highest value of W_{lineage} due to a combination of low genetic diversity (0.005), more basal phylogenetic positioning (Fig. 2), and endemic occurrence outside CUs. The second and third higher values of W were recovered for *A. aff. nactigalli* and *A. wolterstorffi*_6, which have a combination of low values of genetic diversity (0.000) and more derived phylogenetic positions, or higher values of genetic diversity (0.019) and more basal phylogenetic positions, respectively (Fig. 2). The first three lineages are strictly endemic and besides the other 20 lineages (except *A.*

wolterstorffi_4 and *C. aff. nigrovittatus* 1) with the highest values of *W*, do not have recognized populations within protected areas (Table 2). On the contrary, the lowest value *W* is exhibited by *C. melanotaenia*, which presents higher values of genetic diversity (0.093), two populations that occur within CUs and 33 populations encountered outside CUs (Table 2). In this sense, the nine lineages with the lowest values of *W* already present populations occurring within protected areas.

Among the 112 pools that were evaluated for W_{pool} values, the pools of São José do Norte (93), Jaguarão (31), Pedras Altas (43), Cristal (19), and Torres (105) concentrate the highest conservation priority (Table 3). The first of these presents four lineages of annual fish occurring in sympatry (*A. aff. minuano* 1, *A. wolterstorffi_5*, *A. wolterstorffi_6* and *C. melanotaenia*), one of which is strictly endemic (*A. wolterstorffi_6*). The second highest value of W^{pool} was found for pool 31, located at Jaguarão, which is inhabited by two lineages (*A. aff. nachtigalli* and *A. nachtigalli*), one of which is strictly endemic (*A. aff. nachtigalli*). The lowest value of W_{pool} was exhibited by the Viamão (109) pool, which harbors a single lineage of *C. notabilis* that also occurs within the wildlife reserve “Refúgio de Vida Silvestre Banhado dos Pachecos” (Table 3).

Discussion

The biological targets of conservation and their priority of conservation

Decline and extinction of species have often been reported for different animal groups (Ceballos et al. 2017). In this context, it is estimated that up to 50% of current species will be extinct in the next 50 years (Pimm and Raven 2000). Annual fish are certainly among the

most endangered groups (Volcan and Lanés 2018), given both intrinsic characteristics (such as high levels of endemism generally presented by small and isolated populations) and extrinsic characteristics (such as the loss and degradation of their habitats). Nevertheless, given the high levels of cryptic diversity (Costa et al. 2014; García et al. 2015; Costa et al. 2018; Garcez et al. 2018; Fernandes et al. 2021), the frequent incongruences between taxonomic and evolutionary units (Garcez et al. 2018; García et al. 2019; Garcez et al. 2020; Fernandes et al. 2021) and the high levels of differentiation frequently reported even at low spatial scales (Costa et al. 2018; Barbosa et al. 2020), the scenario of vulnerability of annual fish is probably even worse. In this sense, we question here the use of species delimited by traditional taxonomy approaches as the targets of conservation, starting with the definition of independent evolutionary lineages that are considered in most of our further evaluations.

The phylogenetic analyses performed with all specimens from the *A. wolterstorffi* species complex, from the *A. adloffii* species group and from the *Cynopoecilus* genus collected along the PMLS lead to the delimitation of 28 reciprocally monophyletic clusters, of which only ten show an exact correspondence with traditional taxonomic boundaries (*A. adloffii*, *A. arachan*, *A. bagual*, *A. cheffei*, *A. lourenciano*, *A. nigrofasciatus*, *A. pelotapes*, *A. pongondo*, *C. notabilis* and *C. intimus*). Although species splitting was the most common scenario (as for *A. minuano*, *A. nachtigalli*, *A. wolterstorffi* and *C. nigrovittatus*), there were also some cases of taxonomic lumping (as between *A. charrua* and a lineage of *A. minuano* or between some populations of *C. fulgens* and *C. melanotaenia*). All these aspects are further discussed elsewhere (Garcez et al. 2018, 2020; Fernandes et al. 2021). For the sake of this study, besides the subdivision in 28 clusters, a total of 29 evolutionary lineages were considered, given the subdivision of *A. nigrofasciatus* into two lineages that have not yet reached reciprocal

monophyly, as previously reported by Barbosa et al. (2020). Thus, although most of these lineages should probably represent distinct species, some of them may still be considered incipient species or geographically isolated populations. However, regardless of taxonomic status, as they are evolutionarily independent lineages, each of them should be individually considered in the establishment of conservation strategies (Barbosa et al. 2020; Garcez et al. 2018, 2020; Fernandes et al. 2021), which justifies their use in this study.

Nevertheless, unfortunately, genetic data were not available to evaluate taxonomic issues for 13 of the target species (*A. aff. gymnoventris*, *A. camaquensis*, *A. cheradophilus*, *A. cyaneus*, *A. litzi*, *A. luteoflammulatus*, *A. jaegari*, *A. juanlangi*, *A. melanoorus*, *A. paucisquama*, *A. prognathus*, *A. quirogai* and *A. univentripinnis*), which were thus considered as single independent units in the analysis of endemism, sympatry and richness patterns. Considering the entire set of 42 evolutionary lineages/species employed in these evaluations, 19% revealed as strictly endemic, occurring in just a temporary pool. This suggests that several lineages of annual fish will probably be lost in the next years if conservation strategies directed to specific pools are not urgently adopted. In this sense, areas with levels of endemism are irreplaceable and must be urgently conserved since the unique species they contain cannot be saved elsewhere (Mittermeier et al. 2011).

Besides the levels of endemism, there is a lot of concern about the best measures that should be used to assess the level of vulnerability and the conservation status of each taxon (Costa 2012). Traditionally, these estimates are based on: (a) the reduction in population size; (b) the geographic range of the species; (c, d) the estimated population sizes; and (e) a quantitative analysis showing the probability of extinction in the wild (IUCN 2012). Nevertheless, different proposals are now suggesting the use of the phylogenetic position and

the levels of genetic diversity in these evaluations (Lean and MacLaurin 2012). The reasoning behind these suggestions is that basal taxa not belonging to speciose lineages should be prioritized in conservation efforts (Costa 2012), since they comprise a unique fraction of current biodiversity. Otherwise, as the level of genetic diversity of a species or population is directly proportional to its effective population size and to its ability to adapt to a changing environment (Frankham et al. 2004), it probably reflects its chance to persist across time. Thus, we proposed here the use of a new index, named W_{lineage} that simultaneously considers the level of genetic diversity, the phylogenetic positioning, and the number of populations within and outside CUs to measure the priority of conservation presented by each taxon.

According to the values of W_{lineage} thus obtained, the two lineages with the highest conservation priorities share two characteristics: low genetic diversity and occurrence in temporary pools outside protected areas. Moreover, the other 18 lineages with the highest values of W do not have recognized populations within protected areas, except *A. wolterstorffi*_4 and *C. aff. nigrovittatus* 1. Therefore, special attention should be paid to the conservation of all these 20 lineages (*A. arachan*, *A. aff. nachtigalli*, *A. wolterstorffi*_6, *C. aff. nigrovittatus* 2, *Cynopoecilus* sp. 1, *A. lourenciano*, *A. wolterstorffi*_4, *A. cheffei*, *C. aff. nigrovittatus* 1, *A. pelotapes*, *A. bagual*, *C. intimus*, *C. nigrovittatus*, *A. nachtigalli*, *A. wolterstorffi*_5, *Cynopoecilus* sp. 2, *A. aff. minuano* 1, *A. aff. reicherti*, *A. pongondo* and *A. nigrofasciatus*_Pir) which are found in different municipalities (see Table S1). Interestingly, the establishment of lineages based on genetic data proved fundamental in the discovery of 13 of these 20 lineages (Garcez et al. 2018; Barbosa 2019; Garcez et al. 2020), many of which probably encompass new species. This is the case, for example, for *A. lourenciano* and *A. cheffei*, that have been formally described recently (Volcan et al. 2021).

450

451 *Hotspots of diversity of annual fish and their priority of conservation*

452 Of the 144 sampling sites or pools investigated in this study, 55 showed records of sympatry
453 between at least two lineages. When the list of sites with sympatry is evaluated in the context
454 of the list of endemic species, it is possible to observe that five pools harbor endemic lineages
455 and sympatry records, suggesting that their preservation could simultaneously benefit more
456 than one lineage, including a strictly endemic lineage. In fact, the preservation of these four
457 pools could lead to the *in situ* conservation of up to 12 distinct lineages. These results are in
458 agreement with the PAN Rivulídeos (ICMBio 2013), where focal species were defined based
459 on the degree of threat and genetic diversity (in order to protect more basal phylogenetic
460 groups), prioritizing those that live in sympatry.

461 Regarding diversity estimates based on lineage richness evaluated on a broader scale
462 (grids of 1008 Km²), a heterogeneous pattern of distribution of annual fish along the PMLS
463 and adjacent areas could be evidenced. In this sense, while 46% (26 of 56) of the grids
464 presented records of a single lineage, 13% (7 of 56) of them presented records for four or
465 more lineages. The points that presented the highest lineage richness were concentrated along
466 the Southeast (points IX, XIII and XIV) and Southwest (points X and XIV) margins of the
467 Patos Lagoon, as well as the Center-West (points XI and XV) and Southeast (point XII)
468 margins of the Mirim Lagoon. Similar patterns were found by Volcan et al. (2015), in which
469 24 of the 50 municipalities sampled harbored only one species of annual fish. In fact, in both
470 cases, Jaguarão, Pelotas, Rio Grande, Chuí and São José do Norte were among the
471 municipalities with the highest species richness.

472 It is also interesting to note that three of the richest points (IX, XIII and XIV) are

located in the municipality of São José do Norte, where a total of six of the 29 evaluated lineages were found. In addition, this municipality also encompasses three of the pools with the highest levels of sympatry (with up to four lineages being sampled simultaneously) and comprises the distribution area of a strictly endemic lineage (*A. wolterstorffi*_6). These were certainly among the reasons why one of the pools located at São José do Norte (93) recovered the higher value of W_{pool} , allowing us to classify it not only as a hotspot for the diversity of annual fish, but also to categorize it as the point with the highest priority for establishing *in situ* conservation strategies. However, it is important to mention that previous studies have already hypothesized that this area comprises a population mixing zone, potentially resulting from recent flooding (Garcez et al. 2018; Fernandes et al. 2021). Thus, it is possible to question whether at least part of the high diversity of annual fish and the mixture between some pools found in São José do Norte was not indirectly influenced by climate events intensified by anthropogenic actions, like El Niño that affects rain regimes, in this case intensifying them.

Other diversity hotspots are found in the vicinity of the municipalities of Pelotas, Rio Grande, and Jaguarão, broadly agreeing with the results presented by Volcan et al. (2015). Pelotas has record of five lineages and Rio Grande has records of four lineages, and a total of five and nine points of sympatry, respectively. Jaguarão has records of seven lineages, including six pools with sympatry and one endemic specie (*A. aff. nactigalli*). One of the pools (31) located at the municipality of Jaguarão also presented the second highest priority of conservation according to our measures of W_{pool} . On the list of conservation priorities are also pools located at the municipalities of Pedras Altas (43, which harbors the endemic lineage *A. arachan*), Cristal (19, which harbors three lineages, including the endemic *A. cheffeï*) and

Torres (105, which harbors the endemic lineage *Cynopoecilus* sp. 1). Santa Vitória do Palmar and Chuí, on the other hand, embraces three and four distinct lineages and 10 and four points of sympatry, respectively.

In face of a scenario of limited resources, it is recommended that efforts are directed to preserve and restore temporary wetlands (Volcan and Lanés 2018). In this sense, two larger areas are priority: the first located on the east margin (potentially in São José do Norte) and the other on the west margin of the Patos and Mirim Lagoons (potentially among Jaguarão, Pelotas and Rio Grande). This recommendation is based not only on the diversity distribution patterns of annual fish discussed above, but also on the results of phylogenetic and phylogeographic studies conducted with different annual fish species and groups of species in the region (Garcez et al. 2018; Garcez et al. 2020; Fernandes et al. 2021). In fact, a concordant evolutionary pattern, defined by the existence of distinct evolutionary lineages on both margins of the PMLS, has recently been found for *A. minuano* (Fernandes et al. 2021), *A. wolterstorffi* (Garcez et al. 2018) and for the genus *Cynopoecilus* (Garcez et al. 2020). This pattern is justified by the role of the Patos and Mirim Lagoons as historical barriers to the gene flow in annual fish, and has been maintained, at least in some cases, since the initial formation of this large body of water (Garcez et al. 2018).

Additional Challenges

Until this moment, few annual fish species live in protected areas, so most species are not benefited from Brazilian's conservation efforts (Volcan et al. 2015; Volcan and Lanés 2018). Based on the results of endemism, sympatry and species richness presented here, and on the measures of the priority of conservation of lineages, we were able to present a priority order

for the main regions where *in situ* conservation of annual fish species is required. In fact, the creation of Conservation Units in these locations could ensure the conservation of most species registered in the PMLS. This is especially important because most of the localities assessed are located within the Pampa biome, which has the lowest representation in the National Protected Areas System (NPAS) and is considered the second most degraded biome in the country after the Atlantic Forest (MMA 2007; Volcan et al. 2015).

Although two large areas have been highlighted as annual fish hotspots in the Brazilian Pampa, most species show marked endemism and their occupation areas are restricted to a few hectares, usually characterized by patches of isolated water islands in an anthropized rural matrix. Thus, species conservation is closely related to the recognition of their areas of occurrence and the protection of small remnants of habitats, as well as the strengthening of public policies aimed at the conservation of temporary wetlands and a strong plan for environmental education and dissemination of knowledge regarding annual fish and their habitats. In addition, Brazil must act to restore degraded areas, approve laws to protect temporary wetlands and favor public and private investments in scientific research and environmental education (Volcan and Lanés 2018).

In this sense, although the PAN Rivulídeos has led to some small advances and greater visibility of annual fish (ICMBio 2013), much remains to be done to reduce the threats to annual fish. These actions are urging because biodiversity is lost while conservation priorities are being discussed (Costa 2012). As first observed by Costa (2012) for annual fish occurring in Rio de Janeiro, this study confirms that most species of annual fish of the PMLS have few and small populations, that are generally restricted to small areas. Thus, several species are really on the edge of extinction. The threat, in turn, occurs due to several factors, which are

mainly related to weak and negligent public policies regarding Brazilian biodiversity, especially with regard to environmental licensing and the protection of temporary wetlands. Finally, the delimitation of hotspots by municipalities that we have done in this study is of great importance, since environmental licensing is largely done at the municipal level and the distribution limit of a species is often restricted to only one or two municipalities. Therefore, the relationship between species occurrences and municipalities is also important for the delimitation of public conservation policies on a smaller scale, in this case the municipal.

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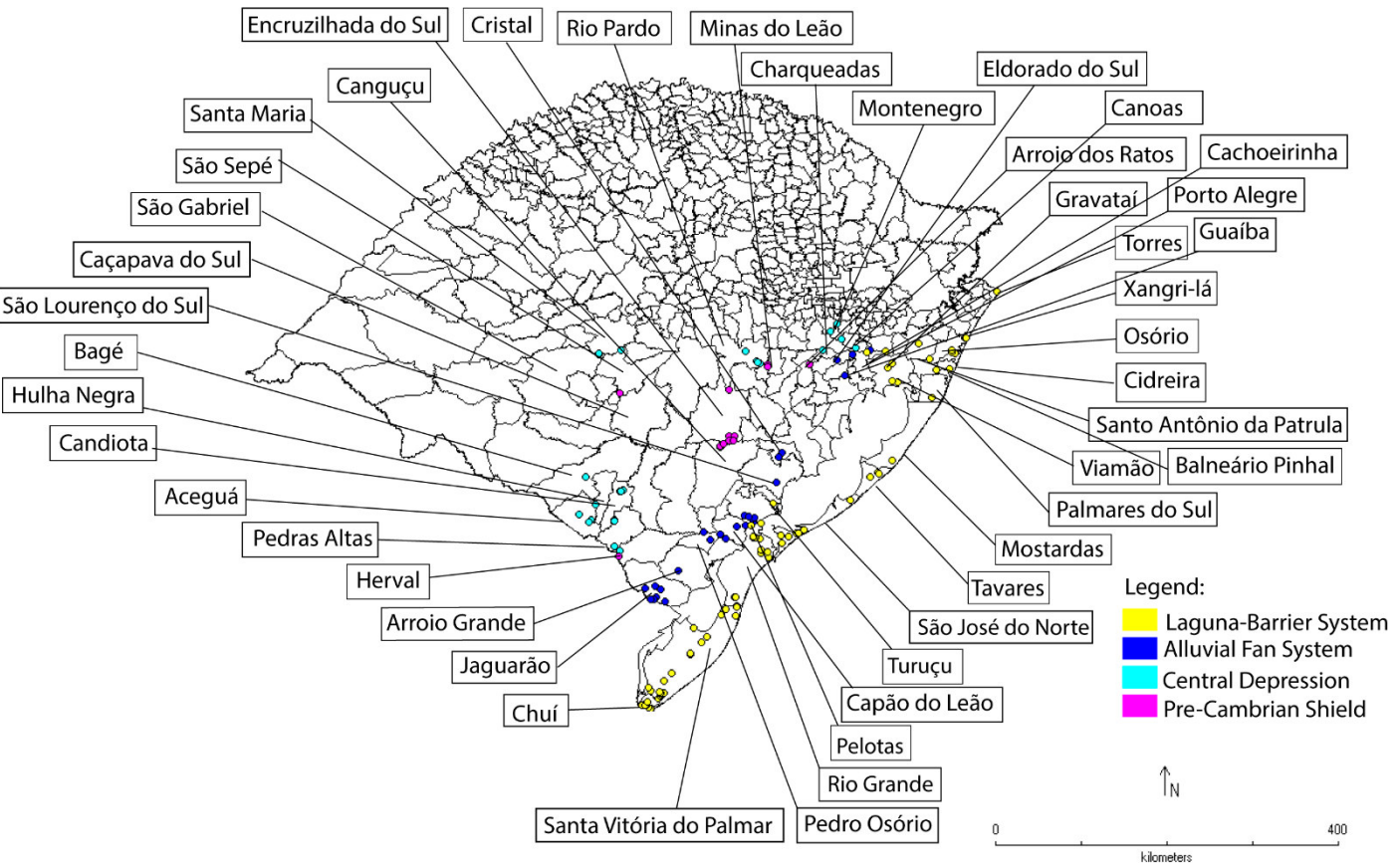
794 **Figure captions**

795 Fig. 1 – Map of the Brazilian Rio Grande do Sul state showing the distribution of the 45
796 municipalities where collections were performed. Some municipalities were sampled in more
797 than one location, totaling 144 collection points or pools. The colors of the symbols represent
798 the different geomorphological units, according to the legend to the right.

799 Fig. 2 – Tree topology generated by Bayesian Analysis for the set of COI and ENC1
800 sequences characterized for the *A. wolterstorffi* species complex, the *A. adloffii* species group
801 and the *Cynopoecilus* genus, depicting the reciprocal monophyly as well as the phylogenetic
802 positioning of each of the lineages evaluated in this study. Numbers above branches refer to
803 the Bayesian posterior probability of each clade. The dotted lines represent the phylogenetic
804 positioning of lineages recovered with an independent analysis performed with the
805 mitochondrial cytochrome *b* gene (Volcan et al. 2021).

806 Fig. 3 – Map of the Brazilian Rio Grande do Sul state showing the distribution of the eight
807 strictly endemic lineages. The captions to the right of the figure represent the colors assigned
808 to each species.

809 Fig. 4 – Map of the Brazilian Rio Grande do Sul state showing the distribution of the patterns
810 of lineages richness along the Patos-Mirim Lagoon System. Colors reflect the number of
811 lineages recovered in each grid according to the legend to the right. Numbers are assigned to
812 each grid with three or more registers, to enhance comprehension of text and Table S1.



814 Figure 1 – Map of the Brazilian Rio Grande do Sul state showing the distribution of the 45
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816 than one location, totalling 144 collection points or pools. The colors of the symbols represent
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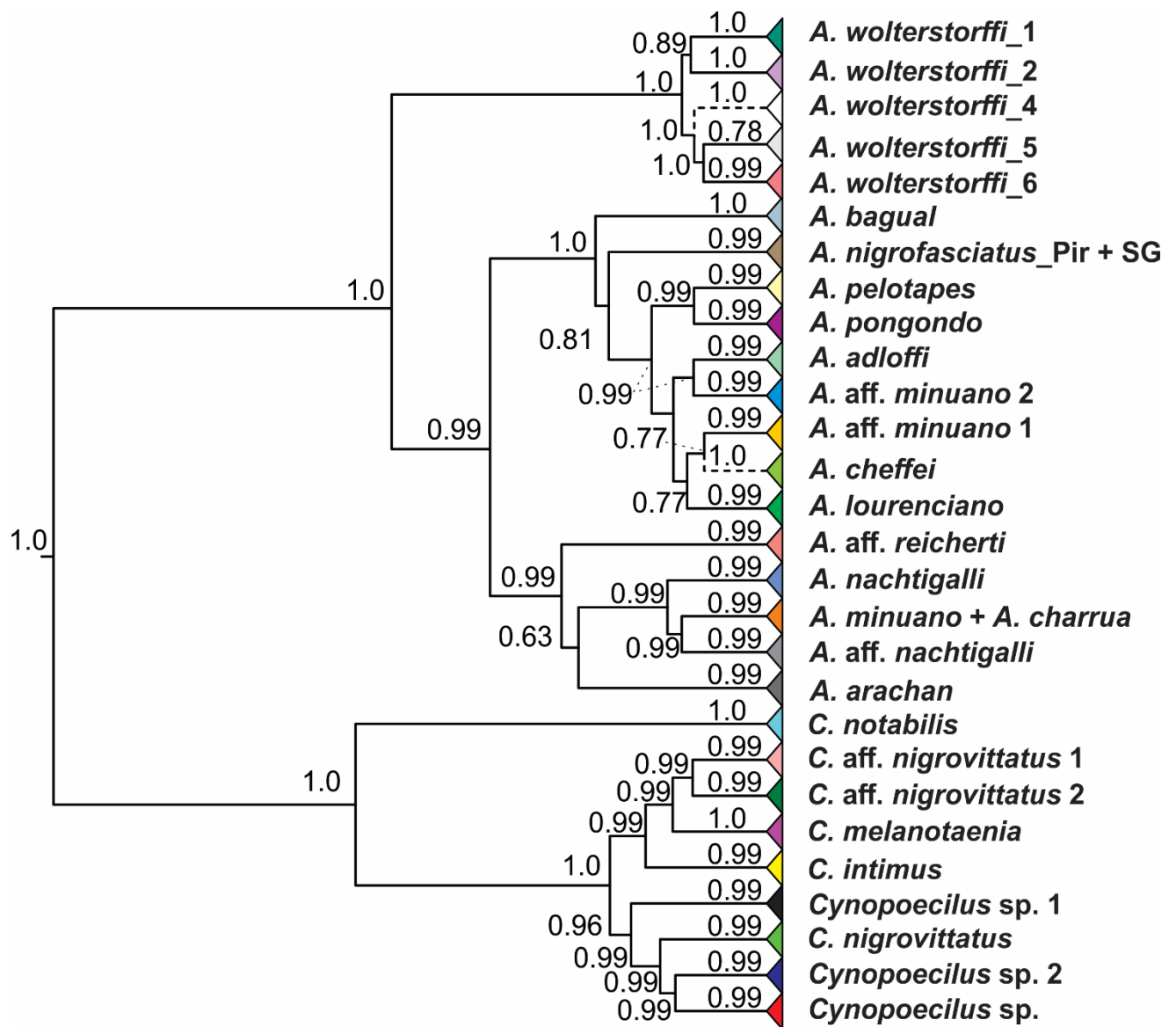
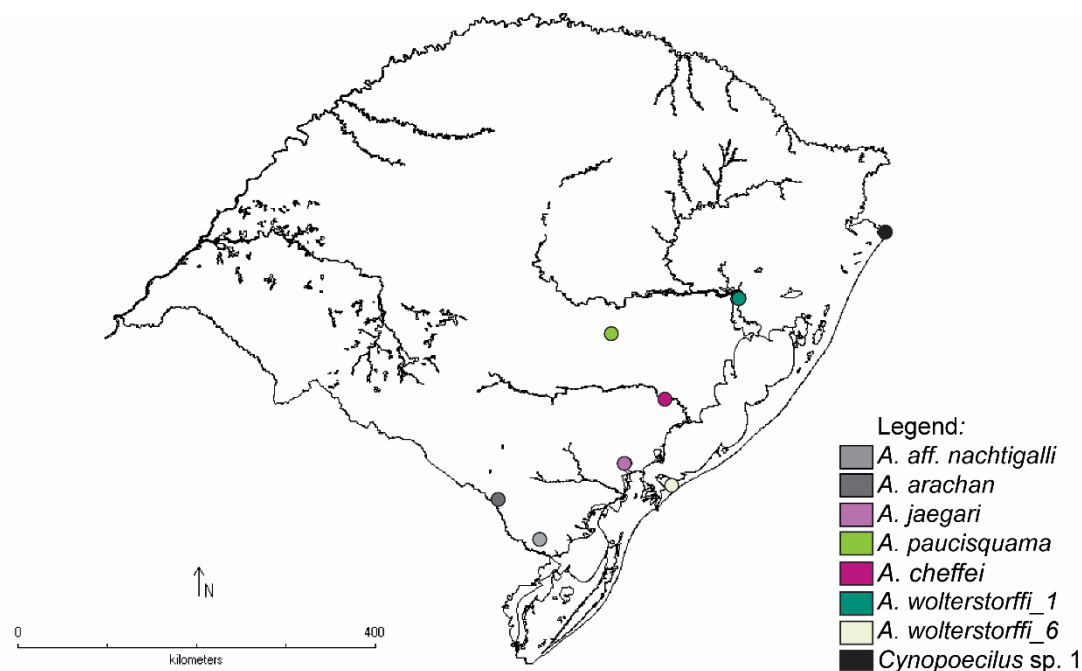


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Figure 3 – Map of the Brazilian Rio Grande do Sul state showing the distribution of the eight strictly endemic lineages. The captions to the right of the figure represent the colors assigned to each species.

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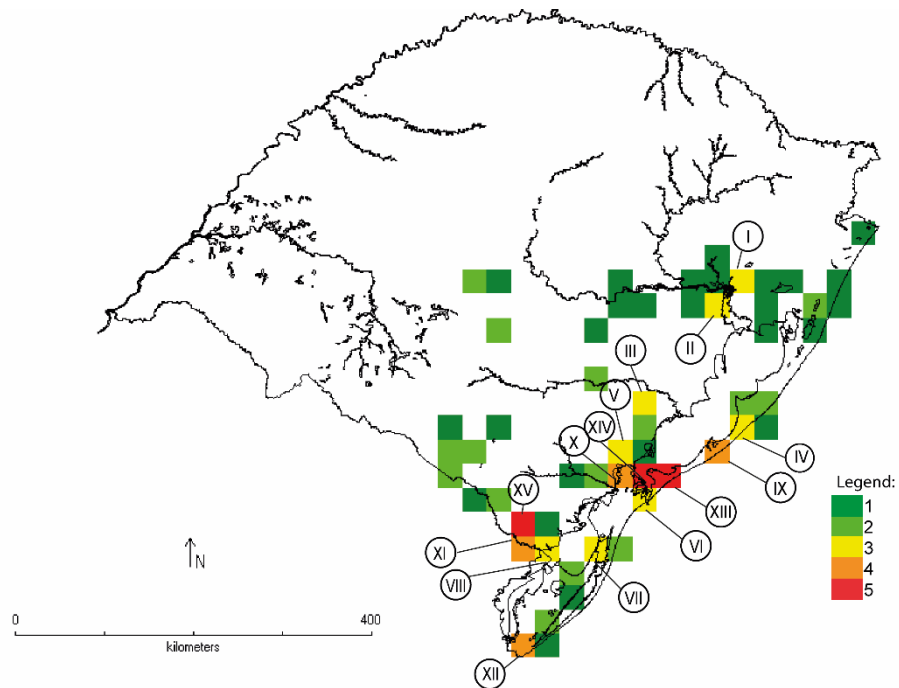


Figure 4 – Map of the Brazilian Rio Grande do Sul state showing the distribution of the patterns of lineages richness along the Patos-Mirim Lagoon System. Colors reflect the number of lineages recovered in each grid according to the legend to the right. Numbers are assigned to each grid with three or more registers, to enhance comprehension of text and Table S1.

853 **Tables**

854 Table 1 – Summary of the dataset used in the establishment of the studied lineages
 855 encompassing the restricted dataset, considering their *a priori* taxonomic delimitation, the
 856 number of sampled localities, the sequences employed for each marker in each analysis and
 857 the corresponding *a posteriori* lineages

Genus	<i>A priori</i> species	Total number of localities	Total number of sequences used in phylogenetic analyzes		Total number of sequences used in diversity analyzes		Lineages defined <i>a posteriori</i>
			COI	ENC1	COI	Cyt <i>b</i>	
<i>Austrolebias</i>	<i>A. wolterstorffi</i>	1	2	2	-	3	<i>A. wolterstorffi</i> _1
		7	4	4	-	10	<i>A. wolterstorffi</i> _2
		2	-	-	-	10	<i>A. wolterstorffi</i> _4
		4	11	8	-	10	<i>A. wolterstorffi</i> _5
		1	2	2	-	3	<i>A. wolterstorffi</i> _6
	<i>A. nigrofasciatus</i>	4	68	16	10	-	<i>A. nigrofasciatus</i> _SG
		4	38	1	10	-	<i>A. nigrofasciatus</i> _Pir
	<i>A. minuano</i>	1	9	3	3	-	<i>A. minuano</i> + <i>A. charrua</i>
		3	42	19	10	-	<i>A. aff. minuano</i> 1
		4	50	25	10	-	<i>A. aff. minuano</i> 2
	<i>A. pongondo</i>	1	13	4	10	-	<i>A. pongondo</i>
	<i>A. arachan</i>	1	10	6	10	-	<i>A. arachan</i>
	<i>A. adloffii</i>	1	1	1	-	9	<i>A. adloffii</i>
	<i>A. bagual</i>	1	15	14	10	-	<i>A. bagual</i>
	<i>A. charrua</i> *	3	16	13	7	-	<i>A. minuano</i> + <i>A. charrua</i>
	<i>A. nachtigalli</i>	1	8	-	8	-	<i>A. aff. nachtigalli</i>

		2	21	-	10	-	<i>A. nachtigalli</i>
		1	9	4	5	-	<i>A. aff. reicherti</i>
	<i>A. pelotapes</i>	1	16	5	10	-	<i>A. pelotapes</i>
	<i>A. aff. reicherti</i>	1	19	-	5	-	<i>A. aff. reicherti</i>
	<i>A. lourenciano</i>	2	5	3	5	-	<i>A. lourenciano</i>
	<i>A. cheffei</i>	-	-	-	6	-	<i>A. cheffei</i>
<i>Cynopoecilus</i>	<i>C. fulgens</i> *	2	12	11	2	-	<i>C. fulgens</i> of type locality = <i>C. melanotaenia</i>
		8	51	46	10	-	<i>Cynopoecilus</i> sp.
		1	3	3	3	-	<i>Cynopoecilus</i> sp. 1
		2	8	5	8	-	<i>Cynopoecilus</i> sp. 2
	<i>C. intimus</i>	1	14	14	10	-	<i>C. intimus</i>
	<i>C. melanotaenia</i>	11	113	106	8	-	<i>C. melanotaenia</i> + type locality of <i>C. fulgens</i>
	<i>C. nigrovittatus</i>	1	15	15	10	-	<i>C. nigrovittatus</i>
		1	21	21	10	-	<i>C. aff. nigrovittatus</i> 1
		1	17	17	10	-	<i>C. aff. nigrovittatus</i> 2
	<i>C. notabilis</i>	3	4	3	4	-	<i>C. notabilis</i>

* Species that present evidence supporting synonymization.

869 Table 2 – Summary of the measures of genetic diversity ($Hd*\pi/\mu$), phylogenetic positioning
870 (number of nodes between the root of the tree and the corresponding taxon), and number of
871 populations within and outside Conservation Units (CU) estimated for each lineage of the
872 restricted dataset, with their respective weights (W_{lineage}) reflecting conservation priority

Lineage	Genetic diversity	Phylogenetic positioning index	Number of pops within CUs	Number of pops outside CUs	W_{lineage}
<i>A. arachan</i>	0.005	5	0	1	243.460
<i>A. aff. nachtigalli</i>	0	7	0	1	228.401
<i>A. wolterstorffi_6</i>	0.019	5	0	1	224.341
<i>C. aff. nigrovittatus 2</i>	0.005	6	0	2	157.563
<i>Cynopoecilus</i> sp. 1	0.214	4	0	1	156.613
<i>A. lourenciano</i>	0	8	0	2	151.592
<i>A. wolterstorffi_4</i>	0.058	4	1	1	144.965
<i>A. cheffe</i>	0.079	9	0	1	140.532
<i>C. aff. nigrovittatus 1</i>	0.025	6	2	1	138.215
<i>A. pelotapes</i>	0	7	0	3	131.144
<i>A. bagual</i>	0.035	4	0	3	131.003
<i>C. intimus</i>	0.017	4	0	4	128.211
<i>C. nigrovittatus</i>	0.027	5	0	3	123.740
<i>A. nachtigalli</i>	0.056	6	0	2	121.437
<i>A. wolterstorffi_5</i>	0.042	5	0	4	104.041
<i>Cynopoecilus</i> sp. 2	0.007	6	0	6	104.019
<i>A. aff. minuano 1</i>	0.025	9	0	3	103.988
<i>A. aff. reicherti</i>	0.089	4	0	4	99.587
<i>A. pongondo</i>	0.027	7	0	8	82.729
<i>A. nigrofasciatus_Pir</i>	0.129	5	0	4	80.701
<i>A. nigrofasciatus_SG</i>	0.130	5	1	3	41.354
<i>A. aff. minuano 2</i>	0.086	8	1	3	37.739

<i>A. adloffii</i>	0.241	8	1	3	26.782
<i>A. wolterstorffi_2</i>	0.225	4	1	7	20.613
<i>C. notabilis</i>	0	2	3	0	12.336
<i>A. wolterstorffi_1</i>	0.041	4	1	0	11.780
<i>Cynopoecilus</i> sp.	0.272	6	1	16	8.662
<i>A. minuano</i> + <i>A. charrua</i>	0.163	7	1	27	7.100
<i>C. melanotaenia</i>	0.093	5	2	33	6.481

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897 Table 3 – List of temporary pools presenting at least one lineage of the restricted dataset, with
898 their respective weights (W_{pool}) reflecting conservation priority

Pool	W_{pool}	Municipality
93	877.701	São José do Norte
31	699.677	Jaguarão
43	486.921	Pedras Altas
19	335.252	Cristal
105	313.227	Torres
98	254.430	São José do Norte
95	223.172	São José do Norte
104	191.366	Tavares
21	176.777	Eldorado do Sul
1	157.563	Arroio dos Ratos
11	157.563	Charqueadas
99	151.592	São Lourenço do Sul
100	151.592	São Lourenço do Sul
27	138.215	Guaíba
49	137.624	Pelotas
50	137.624	Pelotas
51	137.624	Pelotas
23	131.003	Encruzilhada do Sul
24	131.003	Encruzilhada do Sul
25	131.003	Encruzilhada do Sul
6	130.823	Cachoeirinha
5	128.211	Caçapava do Sul
69	128.211	Santa Maria
91	128.211	São Gabriel
101	128.211	São Sepé
34	123.740	Montenegro
35	123.740	Montenegro

36	123.740	Montenegro
2	121.437	Arroio Grande
63	109.823	Rio Grande
29	106.068	Jaguarão
33	106.068	Jaguarão
38	104.019	Osório
39	104.019	Osório
40	104.019	Osório
41	104.019	Osório
90	104.019	Santo Antônio Patrulha
112	104.019	Xangrilá
30	99.587	Jaguarão
32	99.587	Jaguarão
61	89.210	Rio Grande
62	89.210	Rio Grande
64	89.210	Rio Grande
65	89.210	Rio Grande
66	89.210	Rio Grande
67	89.210	Rio Grande
68	89.210	Rio Grande
8	87.182	Capão do Leão
3	80.701	Arroio Grande
44	80.701	Pedro Osório
45	80.701	Pedro Osório
37	76.814	Mostardas
22	69.107	Eldorado do Sul
102	46.402	Tavares
9	41.354	Capão do Leão
10	41.354	Capão do Leão
48	41.354	Pelotas

7	35.444	Canoas
52	35.444	Porto Alegre
46	34.224	Pelotas
60	27.714	Rio Grande
47	20.613	Pelotas
54	20.613	Rio Grande
56	20.613	Rio Grande
59	20.613	Rio Grande
103	18.870	Tavares
15	13.581	Chuí
17	13.581	Chuí
71	13.581	Santa Vitória do Palmar
72	13.581	Santa Vitória do Palmar
78	13.581	Santa Vitória do Palmar
84	13.581	Santa Vitória do Palmar
85	13.581	Santa Vitória do Palmar
87	13.581	Santa Vitória do Palmar
88	13.581	Santa Vitória do Palmar
4	8.662	Balneário Pinhal
18	8.662	Cidreira
26	8.662	Gravataí
42	8.662	Palmares do Sul
92	8.662	São José do Norte
94	8.662	São José do Norte
96	8.662	São José do Norte
97	8.662	São José do Norte
110	8.662	Viamão
111	8.662	Viamão
12	7.100	Chuí
13	7.100	Chuí

14	7.100	Chuí
16	7.100	Chuí
53	7.100	Rio Grande
70	7.100	Santa Vitória do Palmar
73	7.100	Santa Vitória do Palmar
74	7.100	Santa Vitória do Palmar
75	7.100	Santa Vitória do Palmar
76	7.100	Santa Vitória do Palmar
77	7.100	Santa Vitória do Palmar
79	7.100	Santa Vitória do Palmar
80	7.100	Santa Vitória do Palmar
81	7.100	Santa Vitória do Palmar
82	7.100	Santa Vitória do Palmar
83	7.100	Santa Vitória do Palmar
89	7.100	Santa Vitória do Palmar
86	6.790	Santa Vitória do Palmar
20	6.481	Cristal
28	6.481	Jaguarão
55	6.481	Rio Grande
57	6.481	Rio Grande
58	6.481	Rio Grande
106	6.481	Turuçu
107	6.168	Viamão
108	6.168	Viamão
109	6.168	Viamão

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CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A proposta principal da Tese foi auxiliar na delimitação de estratégias de conservação *in situ* de peixes anuais dos gêneros *Cynopoecilus* e *Austrolebias*, através do esclarecimento de questões taxonômicas, micro e macroevolutivas. Os peixes anuais foram escolhidos como grupo de estudo uma vez que os mesmos constituem um dos grupos de vertebrados mais ameaçados do mundo. Isso ocorre tanto por características intrínsecas a essas espécies, relacionadas à ação de um conjunto de condições evolutivas, demográficas e metabólicas, que podem resultar em endocruzamento, baixos níveis de variabilidade genética e na rápida divergência entre populações, quanto por processos extrínsecos relacionados à perda, degradação e fragmentação das áreas úmidas. Dentre os peixes anuais, espécies dos gêneros *Cynopoecilus* e *Austrolebias* foram escolhidas por apresentarem importantes divergências sobre o *status* taxonômico das espécies e por serem endêmicas do Sistema Lagunar Patos-Mirim. Esta área foi escolhida por constituir um *hotspot* de peixes anuais, mas até então sem regiões estratégicas delimitadas para a conservação.

Nosso estudo mostrou que o DNA barcode é uma ferramenta confiável para delimitar espécies ou linhagens de *Cynopoecilus*, especialmente quando utilizada concomitante a um marcador nuclear. Neste âmbito, nossos resultados evidenciaram a subdivisão de *Cynopoecilus* em 8 a 10 grupos bem sustentados, que compreendem diferentes populações das seis espécies descritas anteriormente. No entanto, uma espécie foi considerada inválida (*C. fulgens*), e foram apresentadas pelo menos três novas espécies putativas (*Cynopoecilus* sp., *Cynopoecilus* sp. 1, *Cynopoecilus* sp. 2 e possivelmente *C. aff. nigrovittatus* 1 e *C. aff. nigrovittatus* 2). As duas últimas foram analisadas morfológicamente no capítulo posterior, que confirmou que as mesmas constituem duas populações de uma mesma espécie, aqui nomeada *Cynopoecilus* sp. n. Algumas dessas espécies crípticas provavelmente divergiram a partir de eventos vicariantes, promovidos por grandes corpos d'água como o rio Jacuí e a Laguna dos Patos, embora eventos isolados de dispersão ao longo da última barreira também pudessem ser evidenciados. Em geral, os resultados destacam a necessidade de estratégias de conservação independentes dentro da área de distribuição de cada um desses grupos.

Esses resultados certamente contribuem com o conjunto de evidências que indicam

que a diversidade anual de peixes está atualmente subestimada (GARCEZ et al., 2018; VOLCAN et al., 2015) e que há uma necessidade urgente de mais pesquisas e esforços de conservação direcionados a este grupo (VOLCAN et al., 2015; VOLCAN; LANÉS, 2018). Além disso, a delimitação de novas espécies putativas e o refinamento do conhecimento sobre as áreas de distribuição de cada espécie aumentam a objetividade de estratégias de conservação e aprimoram futuros trabalhos com esses grupos taxonômicos (KEKKONEN; HEBERT, 2014).

Adicionalmente, de modo geral, confirmamos nossas hipóteses iniciais de existência de níveis pronunciados de diferenciação interpopulacional ao longo do sistema lagunar Patos-Mirim, com potencial presença de diversidade críptica dentro de *Cynopoecilus*. Em alguns casos, como para *Cynopoecilus* sp. n., encontramos altos níveis de estruturação populacional dentro de uma mesma espécie. Padrões semelhantes vêm sendo encontrados para diferentes espécies de peixes anuais (GARCEZ et al., 2018; GARCÍA et al., 2009, 2012, 2015; JOWERS; COHEN; DOWNIE, 2007; LOUREIRO; DE SÁ, 2015; PONCE DE LEÓN et al., 2014). No entanto, nossa hipótese de existência de níveis reduzidos de diversidade intrapopulacional não foi confirmada. Provavelmente este resultado esteja relacionado à presença de altas taxas mutacionais em *Cynopoecilus*, o que já foi previamente sugerido para *Austrolebias* (GARCEZ et al., 2018; GARCÍA et al., 2000, 2002). Nesse sentido, novas mutações devem estar surgindo frequentemente, de modo a compensar a variabilidade perdida por deriva genética. Por outro lado, eventos de dispersão e fluxo gênico também foram evidenciados em alguns casos. Para *C. melanotaenia*, por exemplo, existem evidências de pelo menos um evento de dispersão ao longo da Laguna dos Patos, da margem leste para a oeste, bem como uma série de eventos entre populações situadas no sul do Rio Grande do Sul e no norte do Uruguai, em ambos os sentidos.

O segundo capítulo tentou auxiliar no entendimento de questões taxonômicas e evolutivas por meio de avaliações morfológicas e moleculares de indivíduos de *Cynopoecilus* coletados nas bacias do rio Guaíba e do rio Jacuí, a oeste do Sistema Lagunar Patos-Mirim. Esta área apresentava registros para duas possíveis espécies novas (*C. aff. nigrovittatus* 1 e *C. aff. nigrovittatus* 2), que foram confirmadas como pertencentes a uma única espécie do complexo *Cynopoecilus melanotaenia* (*Cynopoecilus* sp. n.). Essa nova espécie é constituída, pois, por duas populações reciprocamente monofiléticas, que devem ter sua conservação

individualmente garantida. *Cynopoecilus* sp. n. se distingue de todas as demais espécies de *Cynopoecilus* pelo maior comprimento do focinho nos machos, pelo número de escamas na série longitudinal nos machos, pelo menor comprimento da cabeça e pelo número de rastros branquiais no primeiro arco branquial. Os resultados encontrados para os marcadores moleculares sugerem uma rota de colonização norte-sul para o complexo *C. melanotaenia*–*Cynopoecilus* sp. n., que ocorreu durante os últimos 150 mil anos. Evidenciamos também a ação do rio Jacuí como barreira entre *Cynopoecilus* sp. n. e *C. nigrovittatus*, que são registradas nas margens opostas do rio. Esta descoberta corrobora o argumento de que a atual diversidade de peixes anuais ao longo do Sistema Lagunar Patos-Mirim é subestimada (GARCEZ et al., 2018, 2020; VOLCAN et al., 2015).

Acerca do estabelecimento de *hotspots* de biodiversidade, podemos constatar que os padrões de riqueza de linhagens de peixes anuais se encontram distribuídos de forma heterogênea ao longo do SLPM, com sete grids apresentando registros para quatro ou mais linhagens. Estes estão concentrados em quatro áreas maiores, localizadas nas margens Sudeste e Sudoeste da Laguna dos Patos e nas Margens Sudeste e Centro-Oeste da Lagoa Mirim, que apresentam vários charcos cuja conservação é urgente. Dentre as 42 espécies ou linhagens avaliadas, oito foram detectadas como estritamente endêmicas, e dentre os 144 charcos amostrados, 55 apresentaram registros de simpatria. Foi possível também desenvolver um novo método de avaliação de prioridade de conservação, tanto para espécies ou linhagens como para áreas, com base no número de populações dentro e fora de Unidades de Conservação (UC), nos índices de diversidade genético e no posicionamento filogenético das espécies. Este método permitiu o estabelecimento de prioridades de conservação para 29 linhagens e 112 charcos com registros de peixes anuais.

Poucas espécies de peixes anuais vivem em áreas protegidas, fazendo com que a maioria das espécies não seja beneficiada pelos esforços de conservação do Brasil (VOLCAN et al., 2015; VOLCAN; LANÉS 2018). Assim, a criação de Unidades de Conservação em pelo menos algumas das localidades avaliadas poderia garantir a conservação da maioria das espécies registradas no SLPM, pois a conservação das espécies está intimamente relacionada ao reconhecimento de suas áreas de ocorrência e à proteção de pequenos remanescentes de seus habitats. De fato, isso é especialmente importante porque a maioria das localidades avaliadas está localizada no bioma Pampa, que possui a menor representação de Unidades de

Conservação e é considerado o segundo bioma mais degradado do país (MMA, 2007; VOLCAN et al., 2015). Assim, o Brasil deve agir para aprovar leis de proteção às áreas úmidas temporárias e favorecer investimentos públicos e privados em pesquisa científica e educação ambiental (VOLCAN; LANÉS, 2018). Como o licenciamento ambiental é em grande parte realizado no nível municipal, a delimitação dos *hotspots* por município que fizemos neste estudo é de grande importância. De fato, a maioria dos processos de tomada de decisão são realizados a nível municipal, e estes capazes de ameaçar ou garantir a proteção de áreas úmidas temporárias.

Por fim, embora a presente Tese tenha auxiliado na delimitação de estratégias de conservação *in situ* de peixes anuais dos gêneros *Cynopoecilus* e *Austrolebias* e no esclarecimento de algumas questões evolutivas e taxonômicas de *Cynopoecilus*, novos desafios surgiram. Entre eles, podem-se citar como principais perspectivas futuras deste trabalho:

- A comparação de novas populações de *Cynopoecilus* com *Cynopoecilus* sp. n., de modo a identificar os limites de distribuição da nova espécie encontrada neste estudo;
- A realização de análises morfológicas e moleculares em outras espécies de *Cynopoecilus* (*Cynopoecilus* sp., *Cynopoecilus* sp. 1 e *Cynopoecilus* sp. 2) a fim de identificar possíveis novas espécies, e, se for o caso, descrevê-las;
- A realização de análises filogeográficas incluindo as espécies e linhagens de *Cynopoecilus* que habitam a margem oeste do SLPM, testando a hipótese de influência dos paleocanais ou do Sistema Laguna-Barreira nos seus eventos de especiação;
- A entrega de um relatório relacionado a distribuição dos *hotspots* de diversidade de peixes anuais ao longo do SLPM para a Secretaria do Meio Ambiente e Infraestrutura do Estado do Rio Grande do Sul, e para as prefeituras das principais cidades onde os *hotspots* estão situados.

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ANEXOS

Anexo 1 – Publicações extras produzidas pela discente no período do doutorado

1. FERNANDES, M. O.; BARBOSA, C.; GARCEZ, D. K.; VARELA JUNIOR, A. S.; VOLCAN, M. V.; ROBE, L. J. Phylogeographic analyses and taxonomic inconsistencies of the Neotropical annual fish *Austrolebias minuano*, *Austrolebias charrua* and *Austrolebias pongondo* (Cyprinodontiformes: Rivulidae). *Environmental Biology of Fishes*, 104, p. 1–14, 2021. <https://doi.org/10.1007/s10641-020-01045-9>
2. CARDOSO, T. F.; GARCEZ, D. K.; RANGEL, E. M.; FERNANDES E SILVA, E. Os desafios do ensino remoto durante a pandemia: relatos de caso na área das Ciências Biológicas em instituições de ensino no extremo sul do Brasil. In: DAMASCENO, M. M. S.; Alves, F. C. (Eds.). *Educação em foco: teoria e prática*. Iguatu: Quipá Editora, 2021 p. 343–355. <https://quipaeditora.com.br/educacao-em-foco>
3. RANGEL, E. M.; MACAGNAN, K. L.; RIBEIRO, L. V.; **GARCEZ, D. K.** Praia do Laranjal: uma visão bioambiental. In: Martins, F. P. (Eds.). **Paisagens do Brasil: dinâmica ambiental, econômica e social**. Iguatu: Quipá Editora, 2021, p. 23–40. <https://quipaeditora.com.br/paisagens-brasil>
4. **GARCEZ, D. K.**; LEON, A. D. O estágio de docência como ferramenta de reflexão. In: BARREIRO, C. B.; LEON, A. D.; KATREIN, B.; FREITAS, L. A. (Eds.). **Formação de professores para a educação profissional e tecnológica**. Pelotas: Editora IFSul, 2020, p. 45–64. <http://omp.ifsul.edu.br/index.php/portaleditoraifsul/catalog/book/180>
5. BARBOSA, C.; **GARCEZ, D. K.**; VOLCAN, M. V.; ROBE, L. J. Evidences for genetic differentiation within the highly endemic and endangered annual fish *Austrolebias nigrofasciatus* (Cyprinodontiformes: Rivulidae). **Journal of Fish Biology**, 96, p. 154–167, 2020. <https://doi.org/10.1111/jfb.14201>
6. FERNANDES, M. O.; **GARCEZ, D. K.**; ACOSTA, I. B.; GHELLER, S. M. M.; CORCINI, C. D.; ROBE, L. J.; VARELA JUNIOR, A. S. Cryopreservation of sperm in

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7. **GARCEZ, D. K.**; BARBOSA, C.; LOUREIRO, M.; VOLCAN, M. V.; LOEBMANN, D.; QUINTELA, F. M.; ROBE, L. J. 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, p. 1503–1515, 2018. <https://doi.org/10.1007/s10641-018-0795-2>
 8. PERAZZO, G. X.; **GARCEZ, D. K.**; TRINDADE TRINDADE, C. R.; PEREIRA, K. M.; TOZETTI, A. M. Is the presence of eggs a relevant cue for predators of freshwater chelonian nests? **Neotropical Biology and Conservation**, 13, p. 350–355, 2018. <https://doi.org/10.4013/nbc.2018.134.10>
 9. Auxílio financeiro aprovado e recebido no valor de R\$ 58.712,09 relativo ao projeto intitulado “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” pela Fundação Grupo Boticário de Proteção à Natureza - número 1090_20171. <https://www.fundacaogrupoboticario.org.br/pt/Biblioteca/Relat%C3%B3rio%20de%20Atividades%202017.pdf>

Anexo 2 – Certificado do Comitê de Ética em Uso Animal

COMISSÃO DE ÉTICA EM USO ANIMAL

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CE

AUTORIZAÇÃO PARA USO DE MAIS ANIMAIS Nº 004/2018

PROCESSO Nº	23118-000183/2015-23
CEUA Nº	P032/2015
UNIDADE	Instituto de Ciências Biológicas
TÍTULO DO PROJETO	Taxonomia molecular, diversidade e estruturação genética de peixes anuais no Rio Grande do Sul (Cyprinodontiformes: Rivulidae)
NÚMERO DE ANIMAIS E VIGÊNCIA	20 indivíduos de cada população – 31/05/2020
ENVIO DO RELATÓRIO FINAL	Abril de 2020
PROFESSOR RESPONSÁVEL	Lizandra Jacqueline Robe

PARECER DA CEUA:

Após a análise de sua solicitação de utilização de mais 60 animais por espécie, realizada em 14 de junho de 2018, o seu pedido foi considerado **APROVADO**, pois cumpre o disposto na Lei nº 11.794, nas demais normas aplicáveis e nas Resoluções Normativas e Diretrizes do Conselho Nacional de Controle de Experimentação Animal (CONCEA). Porém, a aprovação fica **CONDICIONADA** ao envio à CEUA-FURG, pelo responsável do projeto, do aceite ou da ciência da CEUA da Universidade Federal de Pelotas (UFPEL), instituição onde os animais serão eutanasiados.

Rio Grande, 30 de julho de 2018.

Med. Vet. Marcio De Azevedo Figueiredo
 Coordenador da CEUA-FURG

Controle da CEUA - Parecer(es) prévio(s) relacionado(s) ao processo: P004/2016; P032/2016; P045/2016;
 Página 1/1