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

Avaliações filogenéticas e taxonômicas no grupo de espécies de peixes anuais *Austrolebias adloffi* (Cyprinodontiformes: Rivulidae)

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Orientadora: Dra. Lizandra Jaqueline Robe
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Dissertação apresentada ao Programa de Pós-graduação em Biologia de Ambientes Aquáticos Continentais como requisito parcial para a obtenção do título de Mestre em Biologia de Ambientes Aquáticos Continentais.

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Dedico ao meu tio Luiz Antônio (*in memorian*).

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RESUMO

O grupo *Austrolebias adloffi* (Cyprinodontiformes: Rivulidae) compreende 11 espécies de peixes anuais, que vivem em ambientes aquáticos temporários, no sistema lagunar Patos-Mirim, no extremo sul do Brasil e parte do Uruguai. Dentre as espécies do grupo, ao menos seis estão sob risco de extinção, o que se deve principalmente à degradação das áreas úmidas que habitam, das quais são dependentes. O entendimento dos padrões e processos associados à distribuição da diversidade genética se mostra uma ferramenta importante para sua conservação, pois permite, entre outras coisas, definir as unidades evolutivas/taxonômicas independentes que devem ser efetivamente conservadas, bem como os limites de distribuição das espécies. O objetivo deste trabalho foi auxiliar na reconstrução das relações filogenéticas entre as espécies e populações do grupo *A. adloffi*, elucidando questões taxonômicas internas, de modo a contribuir na conservação destas espécies. Para tanto, foram coletados 431 indivíduos do grupo, em 31 localidades, abrangendo as 11 espécies do grupo. Foram amplificados fragmentos dos genes mitocondriais citocromo c oxidase subunidade I (COI) e citocromo b (cytb), e do gene nuclear rhodopsina. Os amplicons foram sequenciados e foram feitas análises filogenéticas, de diversidade e de distância para os marcadores individualmente, para os genes mitocondriais concatenados e para os três marcadores concatenados. As árvores filogenéticas obtidas indicaram a presença de 13 possíveis espécies, sendo esta hipótese corroborada pelos valores de distância par-a-par e pelas redes de haplótipos. A monofilia das espécies *A. adloffi*, *A. viarius*, *A. arachan*, *A. bagual*, *A. pongondo*, *A. pelotapes*, *A. nigrofasciatus* e de *Austrolebias sp* (nova espécie) foi comprovada. Uma população de *A. minuano* e as populações de *A. charrua* apresentam fortes indícios de serem a mesma espécie. Em contrapartida, as demais populações de *A. minuano*, que ocorrem na margem leste da Laguna dos Patos, subdividiram-se em dois clados altamente estruturados entre si, sugerindo a constituição de espécies distintas. Além disso, *A. nachtigalli* também se mostrou polifilética, e uma de suas populações agrupou-se com *A. reicherti*, sugerindo neste caso, ser o primeiro registro de *A. reicherti* em território brasileiro. Por fim, a partir dos dados obtidos, é possível sugerir que a diversificação do grupo se deu de sul para norte, do interior do continente para a costa, padrão diferente do observado para outras espécies do gênero que habitam a mesma região.

Palavras-chave: filogenia molecular; sistema de drenagens Patos-Mirim; killifish.

ABSTRACT

The *Austrolebias adloffi* species group (Cyprinodontiformes: Rivulidae) comprises 11 species of annual fishes, which live in temporary aquatic environments in the Patos-Mirim lagoon region, in the extreme south of Brazil and part of Uruguay. Among the species in the group, at least six are at risk of extinction, which is mainly due to the degradation of the wetlands they inhabit, of which they are dependent. The understanding of the patterns and processes associated to the distribution of genetic diversity is an important tool for conservation, since it allows, among other things, to define the independent evolutionary / taxonomic units that must be effectively conserved, as well as the actual distribution limits of the species. This study aimed to aid in the reconstruction of the phylogenetic relationships between species and populations of the *A. adloffi* group, elucidating internal taxonomic issues, in order to contribute to the conservation of these species. For this, 431 individuals of the group were collected in 31 localities, covering the 11 species of the group. Fragments of the mitochondrial cytochrome oxidase oxidase subunit I (COI) and cytochrome b (cytb) genes, and of the nuclear gene rhodopsin were amplified. The amplicons were sequenced and phylogenetic, diversity and distance analyzes were done for the markers individually, for the concatenated mitochondrial genes and for the three concatenated markers. The phylogenetic trees obtained indicated the presence of 13 possible species and this hypothesis has been corroborated by the values of pairwise distance and the haplotype networks. The monophyly of the species *A. adloffi, A. viarius, A. arachan, A. bagual, A. pongondo, A. pelotapes, A. nigrofasciatus* and *Austrolebias sp* (new species) was confirmed. A population of *A. minuano* and *A. charrua* populations show strong evidence of being the same species. In contrast, the other populations of *A. minuano*, which occur on the eastern margin of the Patos Lagoon, were subdivided into two highly structured clades, suggesting the formation of distinct species. In addition, *A. nachtigalli* was also polyphyletic, and one of its populations was grouped with *A. reicherti*, suggesting the first register of *A. reicherti* in brazilian territories. Finally, from the data obtained, it is possible to suggest that the group diversification occurred from south to north, from the interior of the continent to the coast, a pattern different from that observed for other species of the genus that inhabit the same region.

Key words: molecular phylogeny; Patos-Mirim drainage system; killifish.

APRESENTAÇÃO

Esta dissertação segue o modelo proposto pelo Programa de Pós Graduação e está organizada em três principais partes. A primeira contendo uma introdução geral, abordando os peixes anuais, sua ameaça de extinção devido à degradação dos ambientes aquáticos, e como a biologia molecular (ferramenta deste estudo) auxilia nestes casos. A segunda parte consiste em um manuscrito, o qual apresenta Introdução, Materiais e Métodos, Resultados, Discussão, Referências, Figuras, Tabelas e Apêndices. O manuscrito segue as normas de formatação da revista Journal of Zoological Systematics and Evolutionary Research, para a qual será submetido. A terceira parte do trabalho se refere a uma avaliação geral sobre esta dissertação, levantando a sua importância, bem como perspectivas futuras.

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INTRODUÇÃO GERAL

Biologia molecular e a sua relação com espécies ameaçadas

Segundo o Livro Vermelho da Fauna Brasileira Ameaçada de Extinção (ICMBio, 2018), o Brasil apresenta, atualmente, 1173 espécies sob algum nível de ameaça, das quais 408 são peixes. Apesar da grande representatividade dos peixes nesta lista (34%), este valor pode ser uma subestimativa, devido à ausência de informações importantes sobre várias espécies e ao desconhecimento acerca do número de espécies nos diferentes grupos taxonômicos. Apesar de não serem diretamente utilizados no estabelecimento do status de ameaça das espécies, dados moleculares permitem uma delimitação mais precisa das espécies, bem como de sua distribuição geográfica, fatores importantes na determinação do status de ameaça (Galetti et al., 2009). Através da estimativa da estruturação populacional, por exemplo, pode ser feito um melhor direcionamento dos esforços de conservação, visando um uso mais eficiente dos recursos disponíveis (Matioli e Fernandes, 2012; Beheregaray et al., 2016). Além disso, os níveis de diversidade genética apresentados pelas diferentes populações fornecem estimativas importantes acerca do grau de perturbação do seu biótopo, e da sua própria evolvabilidade (Matioli e Fernandes, 2012). Neste sentido, a compreensão da composição e organização da diversidade genética das espécies é de suma importância, pois fornece uma medida do seu potencial adaptativo e evolutivo (Galetti et al., 2009).

Os níveis de diversidade e estruturação genética apresentados por uma espécie ou população são influenciados por muitos fatores, tais como taxas de mutação, deriva genética, seleção natural e nível de fluxo gênico (Frankham et al., 2004). A deriva genética (mudança aleatória na frequência dos alelos), em especial, apresenta influência pronunciada em populações pequenas. Populações deste tipo também costumam apresentar altas taxas de endocruzamento, o que leva a um aumento nos níveis de homozigosidade dentro da população (Frankham et al., 2004). Outro fator importante a ser considerado é o poder dispersivo dos organismos, o qual pode sofrer influência de barreiras físicas que impedem a migração, favorecendo o isolamento interpopulacional. Nestes casos, além dos altos índices de endocruzamento, há uma redução do tamanho efetivo da população. A união destes fatores tende a diminuir a diversidade genética da população, diminuindo sua capacidade adaptativa (Whitlock, 2000), tanto devido ao colapso mutacional (fixação de alelos deletérios devido a supremacia da deriva genética sobre a seleção natural, que ocorre em populações pequenas), como devido a depressão por endocruzamento (com aumento nos níveis de homozigose para alelos deletérios ou redução dos níveis de heterozigose para loci que apresentam

sobredominância) (Freeland, 2011). No entanto, estes mesmos fatores elevam a divergência entre as populações, podendo culminar em eventos de especiação alopátrica (Ridley, 2006; Matioli e Fernandes, 2012; Beheregaray et al., 2016).

Neste contexto, a filogeografia analisa a distribuição geográfica das linhagens no decorrer do tempo (Avise, 2000), a fim de avaliar os padrões microevolutivos das populações em uma visão espaço-temporal, assim como os fatores responsáveis por estes padrões (Beheregaray, 2008). Porém, uma compreensão completa exige, muitas vezes, a análise dos padrões e processos macroevolutivos, que analisam a evolução focando em alterações a nível de espécie ou acima (Ridley, 2006). Nesse nível de análise, podem ser estabelecidas as relações filogenéticas, permitindo a resolução de questões taxonômicas e a avaliação do cenário espaço-temporal associado aos eventos de diversificação dentro do grupo estudado. O uso concomitante destas duas perspectivas pode, pois, levar a uma compreensão dos mecanismos associados à especiação e às rotas de colonização e dispersão dos mais diversos grupos biológicos, desde as menores até as maiores escalas.

Complementarmente, no âmbito taxonômico, a técnica de DNA-barcode mescla o uso de métodos de análise filogenética com análises de distância, empregando um fragmento do gene mitocondrial citocromo c oxidase subunidade I (*COI*) como marcador (Hebert et al., 2003). Esta ferramenta vem sendo utilizada com sucesso em diferentes grupos, permitindo a identificação de espécies (Hajibabaei et al., 2005; Clare et al., 2007), bem como a descoberta de novas espécies (Costa et al., 2014). Esta perspectiva vem sendo bastante útil na complementação de estudos taxonômicos clássicos, em que os organismos são classificados apenas de acordo com suas características morfológicas. Afinal, quando se compara a forma dos indivíduos com resultados moleculares, nem sempre os dados refletem as mesmas relações filogenéticas, pois muitos caracteres fenotípicos podem ser oriundos de homoplasia ou convergência (Klingenberg, 1996).

Os peixes anuais

Os peixes anuais estão entre os grupos de vertebrados mais ameaçados, representando 30% da ictiofauna ameaçada no Brasil (ICMBio, 2018), e 70% da ictiofauna de água doce ameaçada no Rio Grande do Sul (SEMA, 2014). Esta alta representatividade dos peixes anuais nas listas de espécies ameaçadas se deve, principalmente, à degradação do seu habitat, constituído essencialmente por charcos temporários. O avanço da agricultura, a expansão de áreas urbanas (e consequente poluição química e física), além da construção e duplicação de estradas constituem importantes fatores de redução e fragmentação de áreas úmidas (Maltchik

et al., 2004). Além disso, a maioria destes peixes constituem populações pequenas e isoladas, instaurando um cenário ideal para a atuação de deriva genética e endogamia, o que tende a elevar os níveis de estruturação genética e tornar as populações e espécies ainda mais vulneráveis. Neste sentido, níveis significativos de diferenciação populacional foram recentemente recuperados até mesmo entre populações distando apenas 3 km entre si (Barbosa, 2016). Em outros locais, entretanto, contatos secundários vem sendo sugeridos como uma importante fonte de fluxo gênico entre as populações, levando a proposição de um cenário composto por metapopulações (García et al., 2015).

Diante da vulnerabilidade destas espécies, foi elaborado no Brasil, em 2013, o "Plano de Ação Nacional para a Conservação dos Peixes Rivulídeos Ameaçados de Extinção - PAN Rivulídeos" (ICMBio, 2013). Este documento visa estabelecer mecanismos de proteção aos peixes anuais, através da anulação da perda de seus habitats e do incentivo à realização de estudos técnicos e científicos aplicados à conservação das espécies em foco (ICMBio, 2013). De fato, a importância da realização de estudos sobre estes peixes vem sendo enfatizada já há algum tempo (Reis et al., 2003; Volcan et al., 2009), em vista da escassez de informações que auxiliem na compreensão da biologia dos mesmos, visando a sua preservação. Afinal, estes peixes são de extrema importância para a manutenção do equilíbrio do ecossistema em que se inserem, dado o seu hábito alimentar oportunista, o qual consiste principalmente de invertebrados (Volcan et al., 2011).

Família Rivulidae

A família Rivulidae compreende cerca de 38 gêneros e aproximadamente 428 espécies descritas (Eschmeyer & Fong, 2016), sendo a quarta família mais diversa entre os peixes neotropicais de água doce. A monofilia desta família foi inicialmente sugerida por Parenti (1981), hipótese posteriormente confirmada por diversos pesquisadores através de análises morfológicas e moleculares (Costa, 1990; Murphy e Collier, 1997; Costa, 1998). De acordo com Murphy e Collier (1997), os rivulídeos podem ser divididos em duas linhagens: Cynolebiasinae e Rivulinae. A primeira compreende aproximadamente nove gêneros, incluindo os grupos irmãos *Campellolebias*, *Leptolebias* e *Cynopoecilus*; os grupos irmãos *Nematolebias*, *Simpsonichthys* e *Austrolebias*; e o gênero *Spectrolebias* (Costa, 1998).

Os rivulídeos estão distribuídos ao longo da América do Sul e Central até o sul da América do Norte (Costa, 2008). Na América Central, ocorrem entre o centro do México e Panamá, e ao longo das drenagens do Pacífico na Costa Rica e Panamá. Algumas espécies são endêmicas das Ilhas do Caribe, e táxons tolerantes à água salgada são encontrados da América

do Norte à América do Sul. Na América do Sul, a família é amplamente distribuída na maioria das bacias hidrográficas cis-andinas, do norte da Venezuela até o sul da Argentina, no Brasil e nas drenagens atlânticas trans-andinas da Colômbia e da Venezuela, com algumas ocorrências na bacia costeira do Pacífico no nordeste da Colômbia (Costa, 1998; Costa, 2010). Cerca de um terço das espécies de rivulídeos pode ser encontrada em águas brasileiras, com no mínimo 37 espécies conhecidas para o estado do Rio Grande do Sul. Dentre estas, 31 pertencem ao gênero *Austrolebias* (Costa, 2008; Volcan et al., 2015).

Cerca da metade das espécies de rivulídeos são encontradas em áreas alagadas temporárias que se formam durante períodos de cheias e secam durante alguns meses do ano. Por viverem em ambientes efêmeros, com condições físico-químicas muito variáveis, como baixos níveis de oxigênio e grande amplitude térmica, estas espécies possuem uma série de adaptações características, as quais permitem sua sobrevivência (Volcan, 2009). Neste sentido, muitos rivulídeos apresentam um ciclo de vida curto, que pode se completar em alguns meses, sendo popularmente conhecidos como peixes anuais (Murphy *et al*, 1999).

Durante o período das cheias estes peixes se desenvolvem rapidamente, atingindo a maturidade sexual entre 6 a 8 semanas; se reproduzem continuamente e depositam ovos de resistência no substrato, a profundidades de até 15 cm (Vaz-Ferreira *et al.*, 1966). Pouco antes do charco secar, os indivíduos adultos morrem, devido, provavelmente, às altas temperaturas e aos baixos níveis de oxigênio (Errea e Danulat, 2001). Os ovos entram em estágio de diapausa, havendo uma pausa no desenvolvimento embrionário, que perdura até que o charco encha novamente, quando os ovos eclodem, iniciando um novo ciclo (Podrabsky e Hand, 1999; Arezo et al., 2005).

Gênero *Austrolebias*

O gênero *Austrolebias* Costa, 1998 compreende em torno de 4 espécies de peixes-anuais (Nielsen e Pillet, 2015; Costa et al., 2017), cuja distribuição se estende pelas bacias La Plata e Patos-Mirim, abrangendo sul do Brasil, Paraguai, sul da Bolívia, Uruguai e norte e nordeste da Argentina, além de algumas espécies da Bacia Amazônica (Costa, 1998; Loureiro e de Sá, 2015; Nielsen e Pillet, 2015). A monofilia do gênero é suportada tanto por dados morfológicos (Costa, 2006), quanto moleculares (García et al., 2000; García et al., 2014; Loureiro et al., 2018). Dentre os caracteres diagnósticos do gênero estão: a presença de um processo na porção dorsomedial do palatino; porção dorsal do opérculo igual ou mais estreita que a porção ventral; raios distais e mediais das nadadeiras anal e dorsal levemente ossificados ou completamente cartilaginosos (Costa, 2006).

Enquanto a origem monofilética do gênero é bem suportada, as relações filogenéticas internas são instáveis, com as topologias de árvores variando em função dos dados utilizados (Costa, 2006; García et al., 2014). Apesar disso, a monofilia de alguns clados é bem suportada, sendo a distribuição destes predominantemente alopátrica, indicando um padrão de especiação alopátrica (Loureiro et al., 2015). 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. adloffi*) (Fig. 1). Esta subdivisão vem sendo suportada também por estudos moleculares (García et al., 2002; García, 2006), apesar de análises recentes terem posicionado *A. gymnoventris* e *A. luteoflammulatus* como espécies derivadas, no complexo de espécies denominado *A. gymnoventris-luteoflammulatus*, o qual também inclui a espécie *A. quirogai* (García et al., 2014). Segundo esta hipótese, o gênero se encontra dividido em cinco complexos de espécies, os quais constituem dois clados distintos. O primeiro clado, mais basal, compreende o complexo de espécies *A. alexandri-affinis*; enquanto o segundo clado inclui os demais complexos (*A. bellottii-robustus, A. gymnoventris-luteoflammulatus, A. elongatus* e *A. adloffi-viarius*), como uma politomia basal (Fig. 2). Em ambas as hipóteses filogenéticas a monofilia do grupo de espécies *A. adloffi* foi recuperada. Em um estudo mais recente, o clado mais basal compreende o complexo *a. alexandre-affinis*, seguido por um agrupamento das espécies *A. varzeae* e *A. wichi*; a seguir ocorrem seis subgêneros: *Austrolebias* (compreendendo o complexo *A. bellottii*), *Acrolebias* (grupo *A. adloffi* e a espécie *A. araucarianus*), *Cypholebias* (complexo *A. robustus*), *Megalebias* (complexo *A. elongatus*) e *Acantholebias* (incluindo *A. luteoflammulatus* e *A. quirogai*) (Loureiro et al., 2018) (Fig.3).

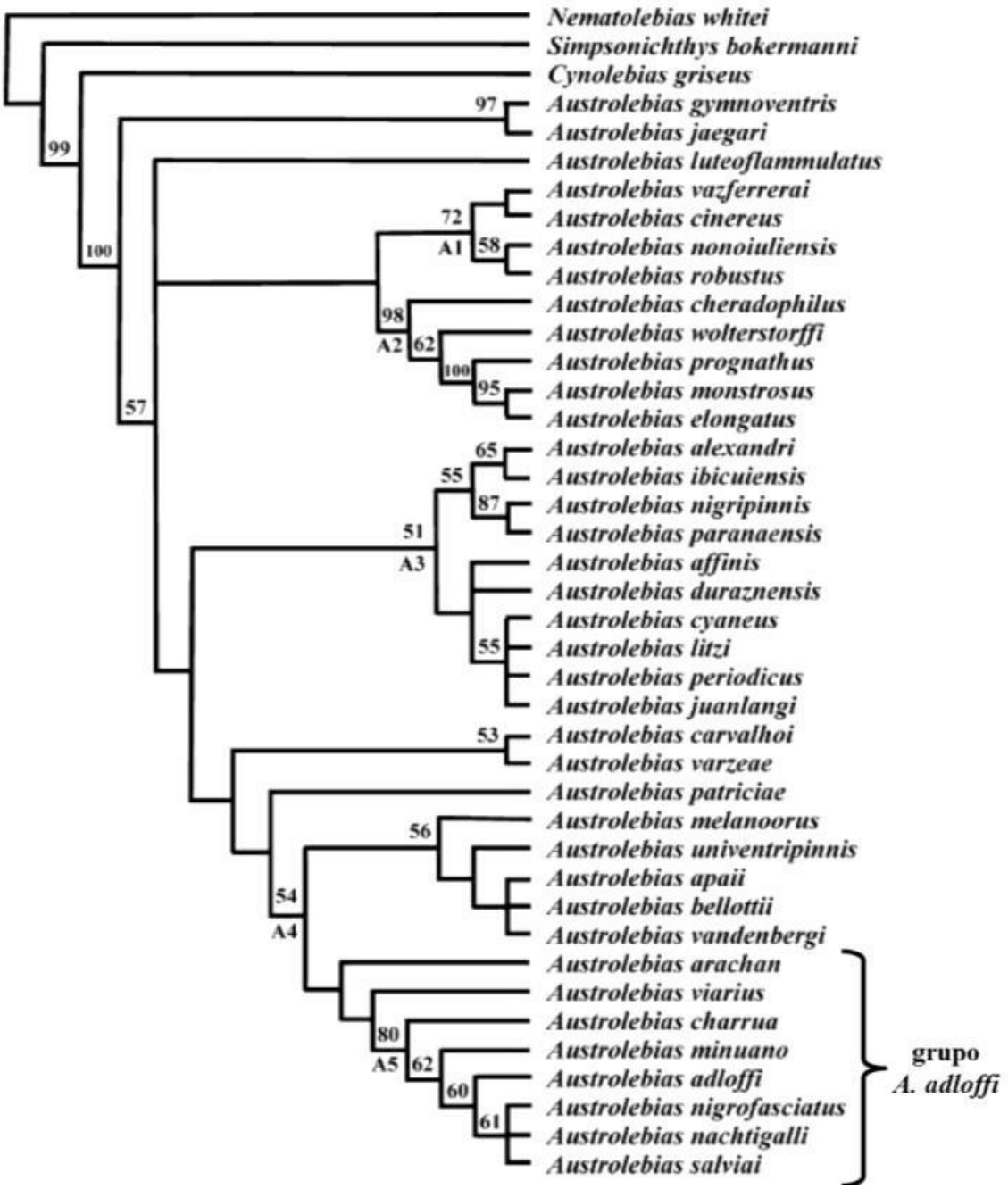

Figura 1 - Árvore de consenso estrito entre dois cladogramas de máxima parcimônia obtidos para diferentes espécies de *Austrolebias* com base na análise de caracteres morfológicos, com o posicionamento do complexo de espécies *A. adloffi* em destaque. Atualmente, *Austrolebias salviai* não é mais considerada uma espécie válida, mas sim uma população de *Austrolebias charrua* (Loureiro e García, 2008). Retirado de Costa, 2006.

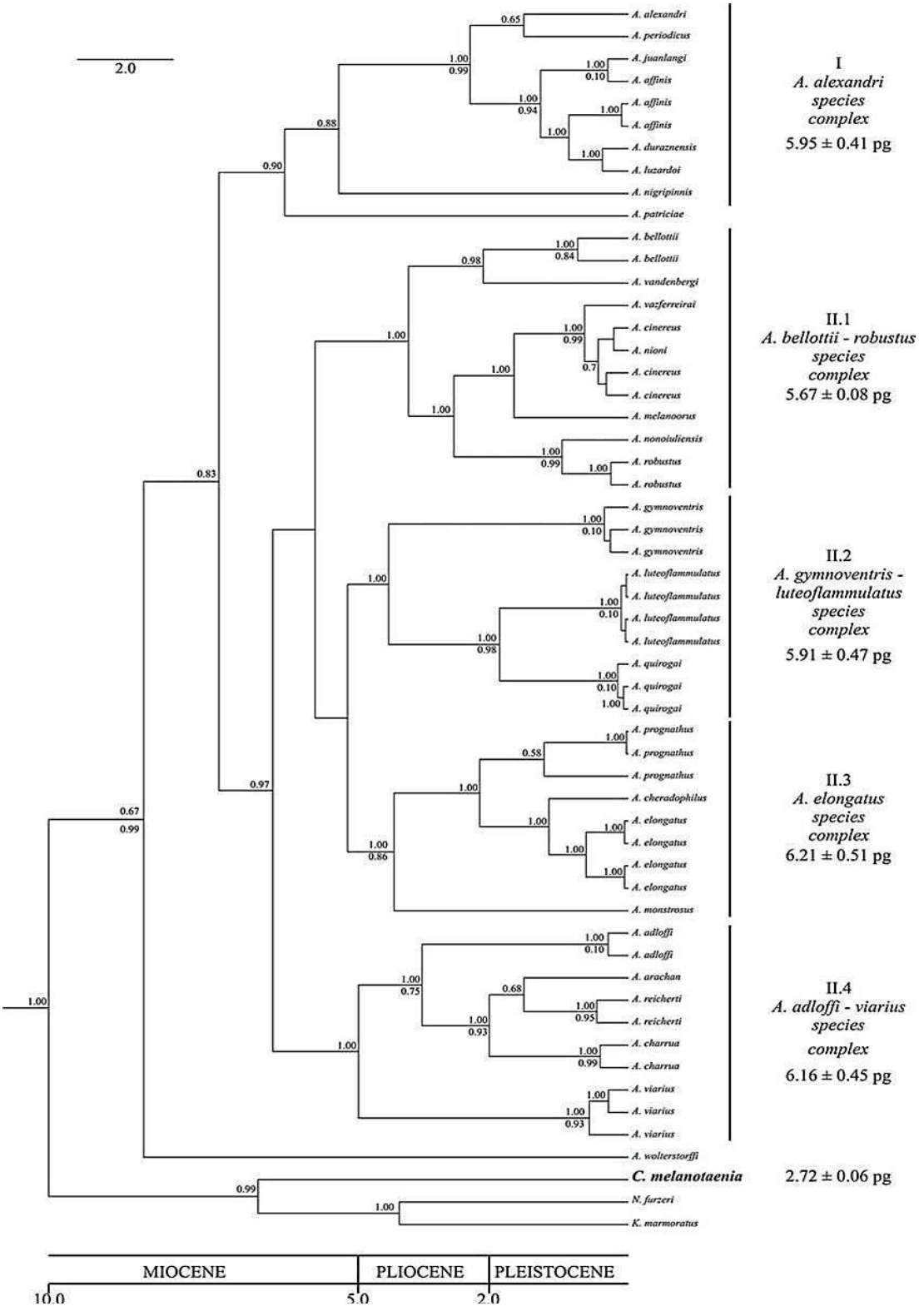

Figura 2 - Topologia da árvore bayesiana reconstruída com base em sequências do gene mitocondrial citocromo-b de diferentes espécies de *Austrolebias* e táxons externos. Números acima dos nós se referem à probabilidade posterior de ocorrência dos clados, enquanto os valores de *bootstrap* estão mostrados abaixo dos nós. Retirado de Garcia et al., 2014.

Grupo de espécies *Austrolebias adloffi*

O grupo de espécies *A. adloffi* compreende onze espécies: *A. adloffi* (Ahl, 1922), *A. viarius* (Vaz-Ferreira et al., 1984), *A. charrua*, *A. minuano*, *A. nigrofasciatus* (Costa e Cheffe, 2001), *A. arachan* (Loureiro et al., 2004), *A. nachtigalli* (Costa, 2006), *A. reicherti* (Loureiro e García, 2008), *A. bagual* (Volcan et al., 2014), *A. pelotapes* e *A. pongondo* (Costa et al., 2017). A distribuição destas espécies se dá ao longo do sistema lagunar Patos-Mirim, abrangendo territórios brasileiros e uruguaios (Costa, 2006; García et al., 2009; Volcan et al., 2014; García et al., 2015). As características que diferenciam este grupo dos demais representantes do gênero são o distinto padrão de cor na porção posterior do pedúnculo caudal, encontrado em fêmeas jovens e adultas e, eventualmente, em machos adultos, que consiste em um par de pontos negros dispostos verticalmente, podendo se unir para formar uma mancha em forma de oito (Costa, 2006). Quanto ao status de ameaça, *A. charrua*, *A. minuano* e *A. nigrofasciatus* constam na categoria "em perigo"; *A. arachan* é considerada "criticamente ameaçada"; *A. adloffi* e *A. nachtigalli* são consideradas "em perigo" a nível nacional, e "criticamente em perigo" a nível estadual (ICMBio, 2018; Rio Grande do Sul, 2014); *A. bagual*, *A. pongondo* e *A. pelotapes* não constam nas listas oficiais da fauna ameaçada por terem sido descritas recentemente (Volcan et al., 2014; Costa et al., 2017); *A. viarius*, *A. reicherti*, *A. arachan* e *A. charrua* também constam como ameaçadas na lista de Especies Prioritarias para la conservación em Uruguay (Uruguay, 2013).

Apesar da monofilia do grupo ser altamente suportada por análises morfológicas e moleculares, as relações filogenéticas dentro do grupo permanecem indefinidas, dado o baixo suporte estatístico, as diferenças amostrais e/ou a incongruência nas relações obtidas por estudos prévios (Costa, 2006; García et al., 2014; Loureiro et al., 2018). Somam-se aqui diversos casos de para ou polifilia, que precisam ser adicionalmente investigados, como por exemplo: 1) a parafilia de *A. charrua* com relação a *A. minuano*, conforme analises preliminares de dados disponíveis no GenBank para o marcador *cytb* (números de acesso: KJ734148.1, KJ734149.1, KJ734150.1, KJ734151.1, KJ734152.1, KJ734153.1, KJ734154.1, KJ734155.1, KJ734156.1, KJ734163.1, KJ734164.1, KJ734165.1, KJ734166.1, KJ475088.1, KJ475089.1, KJ475090.1, KJ475091.1); 2) a polifilia de *A. nachtigalli*, para a qual uma população que ocorre em Jaguarão – RS, Brasil, apresentou maior afinidade com a espécie *A. reicherti* do que com demais populações de *A. nachtigalli* (Loureiro et al., 2018) (Fig. 3). Por fim, existem muitas situações em que evidências preliminares, sejam elas morfológicas ou moleculares, sugeriram a existência, ainda não devidamente testada, de níveis crípticos de diversidade no grupo. Para *A. bagual*, por exemplo, foram detectadas populações

morfologicamente distintas (Volcan, comunicação pessoal), cujo status taxonômico ainda permanece por ser definido. Este fato é agravado em vista da distribuição da espécie, que é restrita a um local altamente impactado pela agricultura, para onde existem projetos de instalação de uma hidroelétrica (Volcan et al., 2014). Uma situação semelhante a esta foi também detectada para *A. nigrofasciatus*, que apresentou populações genética e morfologicamente diferenciadas mesmo sem a amostragem de pontos extremos de distribuição (Barbosa, 2016).

Além disso, a espécie *Austrolebias araucarianus*, descrita recentemente (Costa, 2014) e posicionada no subgênero *Acrolebias* juntamente com *A. varzeae* e *A. carvalhoi* (Costa, 2008), se mostrou geneticamente próxima às espécies do grupo *A. adloffi*, sugerindo a inclusão do grupo no subgênero *Acrolebias* (Loureiro et al., 2018) (Fig. 3). Embora as espécies *A. varzeae* e *A. carvalhoi* sejam encontradas na bacia do Paraná, distantes das espécies do grupo *A. adloffi*, esta similaridade genética aponta para uma relação entre os peixes anuais das bacias do Paraná e do sistema de drenagens Patos-Mirim, sendo esta grande lacuna entre as espécies um possível sinal de extinção local (Loureiro et al., 2018).

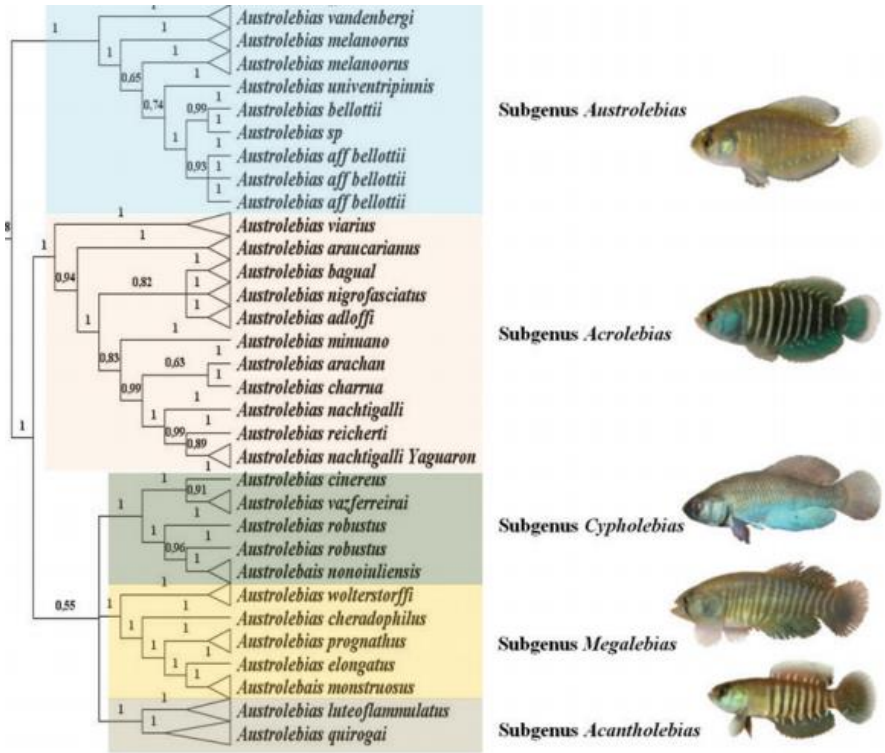

Figura 3 – Árvore filogenética bayesiana do gênero *Austrolebias*, baseado em marcadores moleculares (16S, citocromo b, RAG1, Glyt) e morfológicos. Valores abaixo dos nós se referem à probabilidade posterior. Modificado de Loureiro et al., 2018.

A maior parte das espécies do grupo *A. adloffi* ocorre ao longo do sistema de drenagens Patos-Mirim (Fig. 4). Esta constitui uma área muito rica em espécies endêmicas de peixes-anuais (Costa, 2002), abrigando no mínimo 30 espécies (Volcan et al., 2015), o que está associado a sua abundância de ambientes aquáticos continentais. l

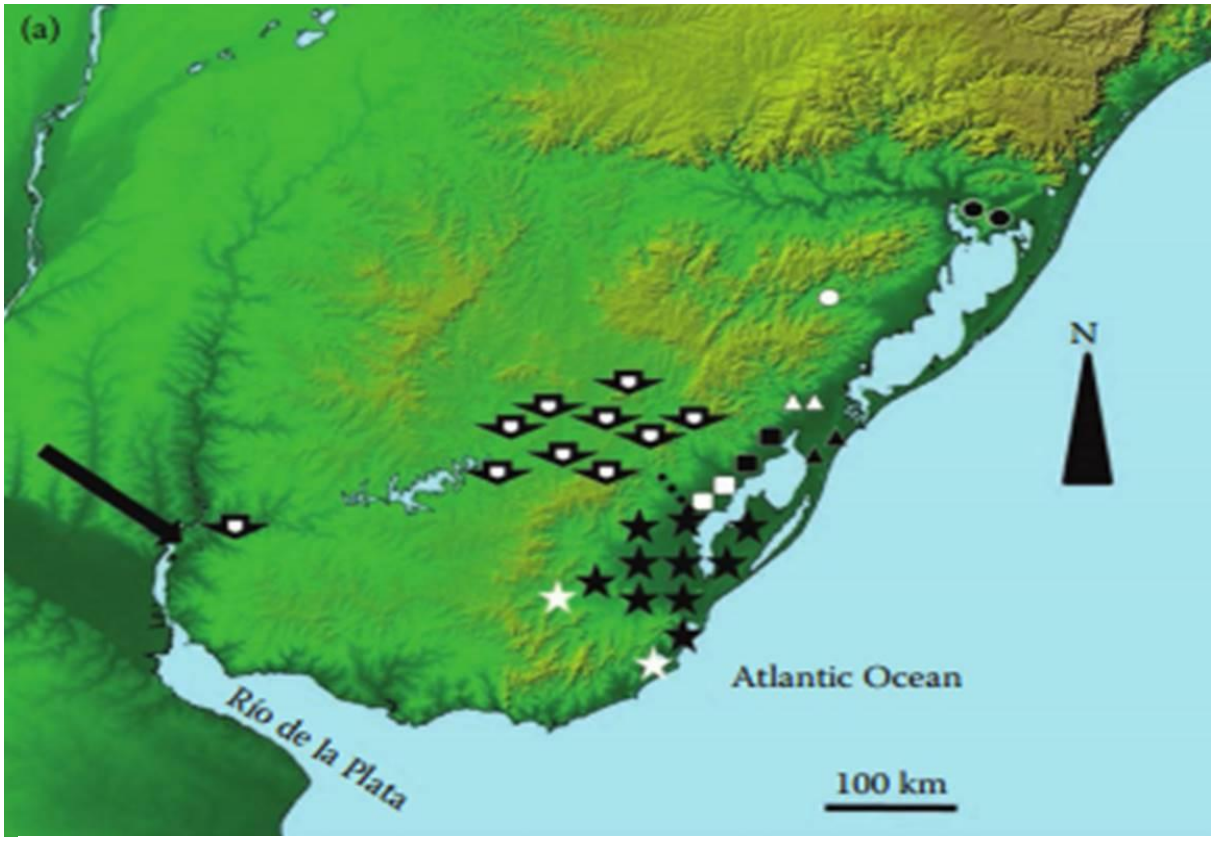

Figura 4 - Distribuição do grupo de espécies *A. adloffi*. Setas com quadrado branco= *A. arachan*; estrelas brancas= *A. viarius*; estrelas pretas= *A. charrua*; quadrados brancos= *A. reicherti*; quadrados pretos= *A.nigrofasciatus*; triângulos brancos= *A. nachtigalli*; triângulos pretos= *A. minuano*; círculo branco= *A. bagual*; círculos pretos= *A. adloffi*. Retirado de Loureiro et al., 2015.

A geologia desta região foi fortemente influenciada pelos eventos de regressão e transgressão marinha que ocorreram durante o Pleistoceno e Holoceno. Tais eventos geológicos foram responsáveis pela formação dos quatro Sistemas Deposicionais Laguna-Barreira (Tomazelli e Villwock, 2005). O primeiro Sistema Deposicional se desenvolveu como consequência do primeiro evento transgressivo-regressivo pleistocênico, há cerca de 400 mil anos atrás, e ocupa os terrenos mais antigos, na região que compreende a Bacia do Paraná e a região de Porto Alegre. O segundo evento transgressivo-regressivo, que ocorreu há aproximadamente 325 mil anos, resultou na formação do segundo Sistema, o qual foi responsável pelo isolamento da Lagoa Mirim. O terceiro Sistema formou-se devido a um terceiro evento transgressivo-regressivo e resultou na implantação final da Lagoa dos Patos,

há cerca de 120 mil anos. O último Sistema Deposicional se desenvolveu durante o Holoceno, decorrente da última transgressão pós-glacial, cujo pico data de cinco mil anos (Tomazelli e Villwock, 2005).

Estudos recentes tem sugerido que a história paleoclimática da região, associada aos eventos de regressão e transgressão marinha, pode ter sido determinante na diversificação de alguns grupos de peixes anuais (Garcez et al., 2018). Para *A. wolterstorffi,* por exemplo, linhagens distintas foram evidenciadas a leste e a oeste da Lagoa dos Patos, com padrões de estruturação compatíveis com a existência de seis grupos populacionais (Garcez et al., 2018), cuja distribuição se assemelha bastante àquela apresentada por diferentes espécies de *A. adloffi.* Além disso, uma alta variabilidade intraespecífica em nível genético, cromossômico e morfológico foi reportada previamente para as espécies do grupo *A. adloffi* que habitam a região (Vaz-Ferreira e Melgarejo, 1984; García et al., 2000; García, 2006).

Considerando a ameaça de extinção das espécies do grupo *A. adloffi*, em face à crescente degradação das áreas úmidas que habitam, pode-se dizer que a realização de estudos genéticos sobre o grupo torna-se especialmente importante. De fato, a reconstrução de sua história evolutiva e a elucidação de suas questões taxonômicas e demográficas pode apresentar um forte impacto no delineamento de estratégias com vistas finais à preservação das espécies. Afinal, o conhecimento sobre diferenças entre as populações é de extrema importância para sua conservação, uma vez que permite avaliar questões taxonômicas, acessar o real status de ameaça das espécies e traçar estratégias efetivas de conservação.

OBJETIVO GERAL

Reconstruir as relações filogenéticas entre espécies de peixes anuais do grupo *A. adloffi*, auxiliando na compreensão do cenário espaço-temporal associado a sua evolução e na elucidação de questões taxonômicas internas ao grupo.

Objetivos específicos

1. Analisar os níveis de diversidade genética de cada espécie do grupo;
2. Inferir os padrões de estruturação entre populações morfológica ou geneticamente diferenciadas nas espécies *A. bagual*, *A. charrua*, *A. nachtigalli* e *A. nigrofasciatus*, determinando seu status taxonômico e assim avaliando a presença de diversidade críptica dentro do grupo;
3. Avaliar a presença de monofilia recíproca nas onze espécies do grupo *A. adloffi*;
4. Inferir as relações filogenéticas entre as espécies pertencentes ao grupo;

5. Avaliar o cenário espaço-temporal associado à origem e diversificação do grupo;

6. Avaliar a congruência entre os padrões espaço-temporais obtidos e a história paleogeoclimática da região por eles habitada;

7. Comparar os padrões macroevolutivos obtidos com os observados para *A. wolterstorffi*, cuja distribuição geográfica é semelhante.

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Phylogenetic evidence to the presence of thirteen taxonomic unities in the *Austrolebias adloffi* species group of annual fish, with species splitting and species merging

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ABSTRACT

The *Austrolebias adloffi* (Cyprinodontiformes: Rivulidae) species group includes 11 species of annual killifishes which live in temporary ponds along the Patos-Mirim drainage system. From these, at least six species are endangered. This study aimed to reconstruct a molecular phylogeny of the group, in order to aid in taxonomic issues, while providing the spatio-temporal scenario of their evolution. For this, 431 individuals were collected in 31 localities, with sampling comprising all species and most populations known for the group. Sequences from the mithocondrial COI and cytb genes, as well as from the nuclear rhodopsin gene were characterized and further employed in phylogenetic and distance analyses. The phylogenetic trees suggested the existence of 13 taxonomic unities, which was supported by the FST, pairwise distances and median joining network. The monophyly of eight species was comproved, one of them comprising a new species of the *A. adloffi* species group. The type population of *A. minuano* seems to be the same species as *A. charrua*. The populations of *A. minuano* from the east coast of Laguna dos Patos presented two diferent clades, comprising two putative new species. *Austrolebias nachtigalli* presented polyphyletic, with one clade comprising the populations of the type locality and surroundings; and the other grouping a single population with *A. reicherti*, which may encompass a single species. Considering the phylogenetic relationships, we can assume that the current area inhabited by the group was established by dispersions from the southern and most internal part of their distribution.

INTRODUCTION

Fishes encompass one of the most endangered taxa accross the world (IUCN, 2018), and in Brasil, just one family of annual fish constitute 39% of the endangered fish species (ICMBio, 2018). These species are included in the Rivulidae Family, which comprises approximately 38 genera and 428 valid species of killifish (Eschmeyer & Fong, 2016), occuring from North America to South America (Costa, 2006). About half of these species live in temporary and isolated wetlands which seasonaly dry and flood, following the precipitation/evapotranspiration ratios. To live in this instable ambient, these fishes have a short life cycle, which is completed in a few months (Murphy, Thomerson & Collier, 1999). These fish grow up fastly during the rainy season, when they reproduce and lay eggs in the substrate (Vaz-Ferreira, Sierra de Soriano & Señorans,1966). As the ponds dry, the adults die due to the déficit of oxigen and the eggs start a diapuse stage, a temporary pause in embryonic development, and this remains until the next rainy season, when the cycle restarts (Podrabsky & Hand, 1999).

However the same peculiarities that allow these fish to live in these environments make them extremely dependent of this biotope (Volcan, 2009). The restricted distribution area, their characteristic life cycle and the crescent degradation of wetlands has put many species under threat of extinction (ICMBio, 2018). This situation is also prompted by other intrinsic characteristics of anual fish, like the isolated distribution and the reduced population sizes, which provides an ideal scenario for strong influence of genetic drift and inbreeding, which reduces the genetic diversity, while increasing the divergence between populations (Galetti *et al*., 2009). The continuous action of these mechanisms can cause allopatric speciation, which further reduces the already restricted distribution occuppied by each species (see, for example, Garcez *et al*., 2018).

The *Austrolebias adloffi* species group comprises 11 species of killifishes: *A. adloffi* (Ahl, 1922), *A. viarius* (Vaz-Ferreira *et al*., 1984), *A. charrua*, *A. minuano*, *A. nigrofasciatus* (Costa & Cheffe, 2001), *A. arachan* (Loureiro *et al*., 2004), *A. nachtigalli* (Costa, 2006), *A. reicherti* (Loureiro & García, 2008), *A. bagual* (Volcan *et al.,* 2014), *A. pelotapes* and *A. pongondo* (Costa *et al.*, 2017). From these, at least seven species are under threat of extinction (ICMBio, 2018). These species are distributed in temporary and isolated ponds along the Patos-Mirim lagoon system, along Brazilian and Uruguayan territories (Costa, 2006; Volcan, Lanés & Gonçalves, 2014; García, Ríos & Gutiérrez, 2015). Although the monophyly of the group is strongly supported (Costa, 2006; García *et al*., 2014; Loureiro *et al.,* 2018), internal relationships remain undefined, due to incongruente or poorly supported relationships. Moreover, some species have not even had their relationships tested. This scenario is further complicated by the presence of some putative para or polyphyletic taxa (Loureiro *et al*., 2018), and by some genetic or

morphological evidence suggesting the presence of cryptic diversity (Barbosa, 2016; Volcan, personal communication).

Considering the threat of extinction of several species of the *A. adloffi* group and the increasing degradation of the wetlands along the Patos-Mirim lagoon system (Volcan, Lanés, Gonçalves & Guadagnin, 2015), genetic studies about the group become especially important in order to assess these taxonomic issues, while addressing the threat status of each species and helping to design effective conservation strategies. Therefore, we aim to reconstruct the phylogenetic relationships among annual fish species of the *A. adloffi* group, helping to understand the spatio-temporal scenario associated with its evolution and elucidating internal taxonomic issues.

MATERIALS AND METHODS

Samplings

This study includes a total of 428 individuals of the 11 species of the *A. adloffi* group, collected between 2015 and 2018, in 31 locations (Figure 1 – for a list of specimens and individual sources, see table S1) distributed along the known distribution range of these species. Samplings were performed after obtaining the collecting license (55651-1) by the Chico Mendes Institute for Biodiversity Conservation (ICMBio), under the Brazilian Ministry of Environment, and were approved by the Ethics Committee on Animal Use (CEUA) of the Universidade Federal do Rio Grande (23116.008163/2015-23 Pq036/2015) (see appendices). The fishes were collected in temporary ponds using a hand net, euthanized with an overdose of 3000mg/L of eugenol and fixed in absolute ethanol. After that, the individuals were transferred to the Laboratório de Genética, Universidade Federal do Rio Grande, Brazil.

Molecular procedures and analyses

Total genomic DNA was extracted from muscular tissue, using a phenol/chloroform protocol (Sambrook, Fritschi & Maniatis, 1989). This DNA was employed in the amplification of approximately 693 pb of the mitochondrial *cytochrome b* (*cytb*) gene, using L14735 (5‟- AAAAACCACCGTTGTTATTCAACTA-3‟) (Wolf, Rentsch & Hübner, 1999) and CB3-H primers (5‟- GGCAAATAGGAARTATCATTC-3‟) (Palumbi, Martin, Romano, Mcmillan, Stice & Grabowaki, 1991); approximately 931 pb of the mitochondrial *cytochrome oxidase subunit I* (*COI*) gene, using LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') (Folmer, Black, Hoeh, Lutz & Vrijenhoek, 1994) and COX1R primers (5-GGYTCTTCRAARGTGTGATAS- 3‟)

(Costa & Amorim, 2011); and approximately 630 pb of the nuclear *rhodopsin* gene, using Rh193 (5‟-CNTATGAATAYCCTCAGTACTACC-3‟) and Rh1039r primers (5‟-TGCTTGTTCATGCAGATGTAG-3‟) (Chen, Bonillo & Lecointre, 2003). PCR conditions were individually optimized (see appendices). To check whether the amplification was sucessfull, the PCR products were subjected to electrophoresis on 0.8% agarose gel, stained with GelRed (Biotium). The amplified fragments were purified with a solution of 7.5M ammonium acetate (C2H7NO2) and sequenced in a Perkin-Elmer ABI Prism 377 Automated Sequencer (MACROGEN, Seoul, Korea), using the same amplification primers.

The obtained sequences had their identify confirmed using BLASTN (NCBI website). The electropherograms were assembled and edited in the Gap4 software of the Staden package (Staden, 1996). Sequences available on GenBank were added to the data matrices, which were aligned under the ClustalW algorithm, as implemented in MEGA 6 software (Tamura, Stecher, Peterson, Filipski & Kumar, 2013). Each polymorphic site encountered along the matrix was individually checked and manually corrected, if necessary. In the nuclear gene, polymorphic sites from heterozygous individuals were replaced according to the degeneracy code, and individual alleles were later unphased using DnaSP 5.10 software (Librado & Rozas, 2009).

Taxonomic units were first assigned under a phylogenetic framework, and later confirmed using a distance approach. In this sense, phylogenetic analyses were performed individually and simultaneously for each marker, by Bayesian Analysis (BA), as implemented in MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2005), with the use of models and partitioning schemes provided by PartitionFinder 2.1.1 (Lanfear, Frandsen, Wright, Senfield & Calcott, 2017). In all cases, sequences obtained for another Rivulidae species (*Austrolebias wolterstorffi*) were used as outgroups. The trees obtained were visualized and edited in FigTree 1.4.4 (Rambaut, 2018). To check the occurrence of cryptic species or even to confirm the need of further synonimization, mean pairwise sequence divergences between groups were measured for each of the three genes (*COI*, *cytb* and *rhodopsin*) in MEGA 6 software (Tamura, Stecher, Peterson, Filipski & Kumar, 2013), using the Kimura-2-parameters (K2P) (Kimura, 1980) substitution model.

The level genetic differentiation between the inferred taxonomic units was also measured through Fst statistics in Arlequin 3.5 software (Excoffier & Lischer, 1993). The relative spatio-temporal relationships between haplotypes were inferred from haplotype networks generated by median-joining in the Network 4.510 software (Bandelt, Forster & Röhl, 1999). In this case, in the *rhodopsin* matrix, a 0 value was atributed for each recombinant site before this reconstruction, looking for the reduction of loops on the haplotype network. These recombinant sites were identified in DnaSP 5.10.

Finally, the level of genetic diversity within each taxonomic unit was evaluated using DnaSP 5.10 software, as estimated from the number of haplotypes (h) or alleles (a) (for mitochondrial and nuclear makers, respectively), values of haplotype diversity (Hd) or expected heterozigosidy (He) (for mitochondrial and nuclear makers, respectively) and nucleotide diversity (π). The same software was also used to check the null hypothesis of neutrality, through the Tajima‟s D (Tajima, 1989), Fu & Li`s D (Fu & Li. 1993), Fu & Li`s F (Fu & Li, 1993) and Fu`s Fs (Fu, 1997) tests.

RESULTS

For the *COI* gene, sequences spanning 931 bp were characterized for 350 individuals, holding a total of 102 different haplotypes. For *cytb*, 693 bp were sequenced for 332 individuals, encompassing 125 haplotypes (Table 1). For *rhodopsin*, 630 bp were characterized for 200 individuals, encompassing 170 alleles (Table 2). As 267 of these individuals presented sequences for both mitochondrial markers, the concatenated mitochondrial matrix spanned 1,624 bp; for the three markers, it was possible to concatenate 141 sequences with 2,254 bp.

Taxonomic subdivision

The phylogenetic trees obtained by Bayesian Analysis, using *COI*, *cytb* and both the concatenated sequences revealed the presente of 13 taxonomic unities, which showed reciprocal monophyly and high values of posterior probabilities (Figs. 2, 3, 4 and 5). The unities obtained comprised nine species individually [*A. adloffi* (presenting sequences solely for *cytb*), *A. viarius*, *A. arachan*, *A. bagual*, *A. pongondo*, *A. pelotapes*, *A. nachtigalli*, *A. nigrofasciatus* and *Austrolebias sp.*], two clusters of species [*A. minuano* (type population, from Leonídeo Island – Rio Grande) + *A. charrua* and *A. nachtigalli aff* (populations of Jaguarão) + *A. reicherti* (presenting sequences solely for *cytb*), and two new clades (*A. aff minuano - SJN*and *A. minuano aff – TAV*). Despite the presence of 55 variable sites in the *rhodopsin* matrix, the phylogeny recovered with this gene revealed completely polytomic, with some punctual clades recovering the grouping of some (but not all) specimens of *A. charrua* and *A. viarius*, *A. pelotapes* and *Austrolebias sp*., *A. charrua* and *A. nigrofasciatus* (see Figure S1).

Concerning the two clusters of species, *A. charrua* revealed paraphyletic with regard to *A. minuano*, and one clade containing some (but not all) *A. charrua* Uruguayan specimens

presented mean distances of 0.001 and 0.008, for COI and cytb, respectively, to another clade containing other *A. charrua* (from Taim and Uruguay) and *A. minuano* sequences (from Leonídeo Island – Rio Grande); for *A. nachtigalli aff* (populations of Jaguarão) and *A. reicherti*, populations revealed reciprocally monophyletic, but mean distances between clades for cytb were as low as 0.006. So, subsequent analysis relyed on the presence of 13 taxonomic unities across our study. Considering this subdivision, the mean COI distance between groups varied from 0.009 (in the comparison between *A. pongondo* and *A. pelotapes*) to 0.119 (in the comparison between *A. viarius* and *A. aff minuano - SJN*). For cytb, the values were somewhat lower, ranging from 0.013 (in the comparison between *A. adloffi* and *A. aff minuano - TAV*) to 0.136 (in the comparison between *A. viarius* and *A. arachan*) (Tables 3 and 5).

As concerns the Fst values recovered individually for each of the three genes, the only comparisons that were not statistically significant occurred between *A. aff minuano - SJN*and *A. adloffi* for *cytb* (Table 4); between *Austrolebias sp.* and: *A. pongondo*, *A. minuano aff. – TAV*, *A. adloffi* and *A. bagual* for *rhodopsin* (Table 5); between *A. bagual* and: *A. pongondo*, *A. minuano aff. – TAV*, *A. nigrofasciatus* and *A. adloffi* for *rhodopsin* (Table 5). For *COI*, all the pairwise comparisons were statistically significant (Table 6). In the significant comparisons, for cytb, Fst values ranged from 0.32 to 0.98, being generally higher than 0.85. Using COI, the values ranged from 0.59 to 1.00, being generally higher than 0.90. For rhodopsin, the values were lower than those recovered for the mitochondrial markers, ranging from 0.12 to 0.90.

The validity of the proposed taxonomic unities was also revealed by the haplotype networks, which detached by the predominance of exclusive haplotypes (Figs. 6, 7 and 8). In fact, only one haplotype was shared among the groups *A. aff minuano - SJN*and *A. aff minuano – TAV* for *COI* and *cytb* genes. In this sense, taxonomic unities presented a restricted and isolated distribution along the network, and all of them encompassed different haplogroups. These haplogroups were separated by a minimum of three mutational steps.

Phylogenetic relationships and diversity measures

Along the different resolved phylogenetic trees (Figs. 2, 3, 4 and 5), *A. viarius* was recurrently recovered as the early offshoot within the *A. adloffi* species group. After this divergence, the remaining species split off to constitute two independent lineages: one encompassing *A. arachan*, *A. nachtigalli*, *A. reicherti*, *A. minuano* + *A. charrua*, and the other

grouping *A. bagual*, *A. pongondo*, *A. pelotapes*, *A. minuano aff – SJN*, *A. minuano aff – TAV*, *A. adloffi*, *A. nigrofasciatus* and *Austrolebias sp*. Interestingly, these lineages share some geographic idiossyncrassies, with species with more interior distribution (as *A. arachan* and *A. bagual*, respectively) encompassing the early offshoots (at least as COI tree is concerned – Fig. 2). Besides this, none major geographic structure could be detected, except for a clear pattern of isolation by distance, where geographically closer species are also phylogenetically closer (as is the case for *A. pongondo* and *A. pelotapes*, *A. charrua* and *A. minuano*).

Table 1 shows the results of the diversity and neutrality tests implemented for each taxonomic unit using both mitochondrial markers individually, whereas Table 2 shows these results for the nuclear marker *rhodopsin*. In general the haplotype diversity values (Hd) and the expected heterozigozity were generally higher than 0.6 for all markers, although *A. arachan* has shown smaller values for *cytb* and *rhodopsin*. The nucleotide diversity values (π) were generally low, ranging from 0.00035 to 0.02528. For almost all species/groups, the neutrality tests were not statistically significant ($p > 0.05$), except for *A. minuano + A. charrua* and *A. nachtigalli aff. + A. reicherti* using *COI* and for *A. nigrofasciatus* using *cytb*.

DISCUSSION

This study reinforce the validity of the species *A. adloffi*, *A. viarius*, *A. arachan*, *A. bagual*, *A. pongondo*, *A. pelotapes*, and *A. nigrofasciatus,* which revealled reciprocallly monophyletic with high posterior probabilities, and presented high values of FST and pairwise mean distances in regard to the other sampled taxonomic units. The species distribution on the median-joining network reinforces the validity of these species.The monophyly of most of these species, as *A. adloffi, A. viarius* and *A. arachan* has been found in previous studies (García et al., 2014; Loureiro et al., 2018). Nevertheless, this is the first study including all the species of *A. adlofii* group.

Austrolebias nachtigalli raises a doubt about the real geographic distribution of the species, and suggests some cases of synonymization added to other cases of additional subdivision. In this sense, although some specimens of the population from Arroio Grande [type locality of *A. nachtigalli* (Costa, 2006)] added to somes specimens of the population Juncalzinho seem to be monophyletic, as supported by posterior probability, FST, pairwise mean distance and median-joining network, its branching follows that of another clade encompassing other members of the Juncalzinho population. Moreover, the population of Jaguarão river basin branches out in another lineage, revealing more closely related to *A.*

reicherti. This last situation was also observed by Loureiro et al. (2018), using different molecular markers. As the clade including *A. reicherti* and *A. nachtigalli* from Jaguarão presented a well supported reciprocal monophyly in all the performed phylogenetic analyses, and distance values comparing both species were generally low, we agred that we were sampling three populations of the same species. In this sense can be assumed that this is the first register of *A. reicherti* in Brazil, and this way we called the populations of Jaguarão as *A. reicherti*. As individuals of *A. nachtigalli* from the type locality grouped in an independent branch, we maintained these as *A. nachtigalli*. This strategy is further supported by the fact that, despite the disjunct distribution, characters that differentiate these two species have not been reported (Loureiro & García, 2008).

Another curious situation was revealed by the populations of *A. minuano*, which were divided into three independent clusters: one presenting individuals of the Leonídeo population [type locality of the species (Costa & Cheffe, 2001)] intermingled with individuals from all populations of *A. charrua*; and the other two encompassing the sister species of *A. sp* and *A. adloffi*, respectivelly. The first of these results suggest that *A. minuano* of the type population, occurring on the West margin of the Patos Lagoon, and *A. charrua* could be a single species, an issue raised earlier by Loureiro et al. (2018). More studies are needed, but if these species are synonimized, according to the taxonomic norms, they would be called *Austrolebias minuano*, constituting the first record of *A. minuano* for Uruguay. On the other hand, the independent branching, the considerable high values of FST and pairwise mean distances, and the disjunct distribution of the other two clades, which occur in the East coast of the Patos Lagon, suggest that we are dealing with other two species of the *A. adloffi* group which share one haplotype for *COI* and *cytb*. These were called as "*A. minuano aff – SJN"* and "*A. minuano aff – TAV*" in allusion to the populations of São José do Norte and Tavares municipalities, respectively, and clustered with *A. adloffi* and *A. sp,* respectively. The grouping of some populations of the eastern coast of the Patos Lagoon with *A. adloffi*, as recovered here for cytb, was already reported by Loureiro et al. (2018), although the population of *A. sp* had not been previously sampled. The maintenance of the independent status of these four species is, nevertheless, sustainable given the mean distances higher than 0.01 between them and they respective sister species.

In this sense, the populations called as "*Austrolebias sp"*, from São Lourenço do Sul – RS, Brazil seems to be a new species of the group. Altough the number of specimens here sampled is small, the results present high statistical support. The new species is shown as sister to *A. minuano aff -SJN* from the east coast, whith high values of posterior probability to

its reciprocal monophyly. The FST values for *COI* ranged from 0.59 (comparing with *A. aff minuano - SJN*) to 1.00 (comparing with *A. pongondo*), with most of the values higher than 0.92. The network reinforces the validity of the new species, which is seen as an independent haplogroup that does not share any haplotypes with other species.

So, we are confident that we are dealing not with 11, but with at least 13 species of the *A. adloffi* species group. This result comes with several nomenclature changes, some of which resulted in the proposition of species with a very restricted distribution (although in the cases of *A. reicherti* and *A. charrua*, distribution was somewhat expanded). Even so, the results of diversity presented high values of haplotype diversity and expected heterozigozity, contrasted with low values of nucleotide diversity for all the taxonomic unities. That was already expected from comparisons with other studies performed with annual fish sampled at the same region (Barbosa, 2016; Garcez et al., 2018). Moreover, considering that these fishes live in small and isolated populations, that are subject to strong influence of evolutive mechanisms which tend to reduce the genetic diversity of this organisms (Frankham, Ballou & Briscoe, 2004), low diversity values are not at all unexpected. In fact, a single case of mitochondrial haplotype sharing was detected among all the taxonomic units here considered, evidencing that both high and small water bodies, and even terrestrial áreas, are acting as effective barriers to the dispersion of these fish.

As concerns the phylogenetic relationships between the taxonomic unities, this study strongly supported *A. viarius* as the first to branch off, followed by two clades comprising all the other species. The early offshoot of *A. viarius* was previously observed by García *et al*. (2014) and by Loureiro *et al*., (2018), both using molecular markers, but differs from the morphologic phylogeny proposed by Costa (2006), which presented *A. arachan* as the first divergence within the group. The first subsequent clade comprises *A. nigrofasciatus*, *A. bagual*, *Austrolebias sp.*, *A. minuano aff – SJN*, *A. minuano aff – TAV*, *A. adloffi*, *A. pongondo* and *A. pelotapes*, and is somewhat similar to the polytomic clade grouping *A. bagual*, *A. nigrofasciatus* and *A.adloffi* as a polytomy recovered by Loureiro et al. (2018), considering the wider sampling of this study. The second clade comprises *A. arachan*, *A. reicherti*, *A. nachtigalli* and *A. minuano+A. charrua.* Although *A. arachan* was previously presented as sister to *A. reicherti* (García *et al.*, 2014), or to *A. charrua* (Loureiro *et al*., 2018), to the exception of the analyses performed individually with *cytb*, most of our results supported it as the early offshoot of the second clade. So, in general, the division in two clades is congruent, but can not be deeply compared with previous studies due to diferences related to the number of species analyzed (some species had not even been descripted in older studies) (Costa,

2006; García *et al*., 2014) or to the number of populations and individuals sampled for each species.

When these phylogenetic relashionships are associated to the geographic distribution of the species, it can be assumed that the diversification of the group has started in the southern of South America, with further diversification to the north and coast of the distribution area. This can be inferred by the early offshoot of *A. viarius* (which occurs in Uruguayan territories) in the major clade of the *A. adloffi* group, and by the putative early offshoot of *A. bagual* and *A. arachan* (both of which present more interior distributions) in the two minor internal clades. These two clades seems to have diversified independently, the first comprising mainly the region of Rio Grande and Pelotas (southern Brazil) and some populations inhabiting the east coast of the Patos Lagoon, while the other encompasses mainly species from interior regions of the state and populations inhabiting the proximity of the Mirim Lagoon. The phylogenetic relationships seem to be deeply related to the geographic distance between species. *Austrolebias nigrofasciatus*, for example, are distributed next to *A. pongondo* and *A. pelotapes*, and this was reflected on the phylogenetic trees.

Interestinly, *A. aff minuano - SJN*and *A. minuano aff – TAV*, which occurr in the East coast of Patos Lagoon also branch in the same clade as *A. nigrofasciatus, A. pongondo* and *A. pelotapes*, which occur in the West coast of this Lagoon. Considering that rivers and other large watercourses have been proved as effective barriers to gene flow of annual fish (Bartáková, Reichard, Blazek, Polacik & Bryja, 2015), it is possible that this divergence resulted from a vicariance event associated with the Lagoon-Barrier depositional systems that occurred in the region during Pleistocene and Holocene (Tomazelli & Villwock, 2005). In this case, nevertheless, at least a single event of regress to the West coast should have occurred, given the grouping of *A. aff minuano - SJN*with *A. sp.* These putative migrations (either from the west to the east, or vice versa, might have occurred due to waterfowl, that carried out eggs from one side of the lagoon to the other. In fact, the waterfowl are proving capable of transport (and disperse) many aquatic organisms, as well as viable fish eggs (Figuerola, Green & Santamaría, 2003; Volcan, personal comunication). Nevertheless, as for *cytb A. adloffi* (which inhabits the northernmost part of the distribution of the group, occurring in the north coast of the Patos Lagoon) is also nested in this clade, it is possible that the eastern coast was first colonized from a stepwise migration along the margin of the Lagoon. Anyway, this scenario differs from that inferred for other rivulidae species which have similar distributions, as is the case for *A. wolterstorffi,* which presented diversification from north to south and encompassed two lineages occurring at the East and West of the Patos Lagoon, respectively

(Garcez *et al*., 2018). In this sense, more studies are necessary, with the use of chronophylogenetic analyses allowing to infer the time of divergence between the species of the group, and thus to relate these results with the geographical formation of the region.

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TABLES

Table 1 - Genetic diversity and neutrality tests performed for each taxonomic unity using *COI* and *cytb* mitochondrial markers.

Molecular marker	Species/groups	N	h	Hd/sd	π/sd	Tajima's D	Fu and Li's D	Fu and Li's F	Fu's Fs
COI	*A. viarius*	4	2	0.667±0.04167	0.00072±0.0000000	1.63299	1.63299	1.27657	0.540
	A. minuano + A. charrua	32	12	0.728±0.00585	0.00668±0.0000032	-2.26954*	-3.74192*	-3.84374*	0.114
	A. nachtigalli	29	6	0.692±0.00403	0.00783±0.0000014	0.87068	0.29191	0.55984	6.352
	A. reicherti	29	8	0.717±0.00688	0.01027±0.0000554	-2.62943*	-4.97049*	-4.95748*	4.202
	A. arachan	10	2	0.356±0.02532	0.00038±0.0000000	0.01499	0.80424	0.68403	0.417
	A. bagual	15	4	0.762±0.00440	0.00153±0.0000001	0.51700	1.15208	1.12422	0.472
	A. pongondo	13	1	-	-	-	-	-	-
	A. pelotapes	16	2	0.325±0.01565	0.00035±0.0000000	0.15575	0.68829	0.62728	0.551
	A. minuano affinis – SJN	56	19	0.832±0.00180	0.00864±0.0000011	-0.09053	-1.04942	-0.83263	-0.733
	A. minuano affinis – TAV	36	12	0.857±0.00191	0.00177±0.0000001	-1.64325	-2.43512	-2.56532	-7.393
	A. nigrofasciatus	106	34	0.919±0.00029	0.00378±0.0000000	-1.29072	-1.23127	-1.50383	-21.126
	Austrolebias sp.	5	1	-	-	-	-	-	-
cytb	*A. viarius*	7	6	0.952±0.00912	0.01498±0.0000111	0.09825	-0.01313	0.01311	0.216
	A. minuano + A. charrua	38	11	0.815±0.00269	0.00482±0.0000005	-0.99350	-0.71541	-0.95395	-1.176
	A. nachtigalli	26	7	0.735±0.00371	0.02528±0.0000089	1.09299	0.52788	0.83282	11.450
	A. reicherti	33	6	0.604±0.00729	0.00581±0.0000007	0.83239	0.59181	0.78435	3.241
	A. arachan	15	5	0.695±0.01192	0.00220±0.0000001	-0.03052	-0.26173	-0.22893	-0.477
	A. bagual	12	8	0.909±0.00421	0.00383±0.0000003	0.56310	0.28246	0.40206	-2.980
	A. pongondo	19	5	0.673±0.00807	0.00204±0.0000001	-0.03341	-0.38689	-0.33286	-0.301
	A. pelotapes	14	8	0.890±0.00364	0.00392±0.0000003	0.29780	0.35780	0.39068	-2.261
	A. minuano affinis – SJN	22	17	0.974±0.00047	0.01912±0.0000032	0.69659	-0.32366	-0.01397	-2.691
	A. minuano affinis – TAV	31	18	0.948±0.00058	0.00511±0.0000007	-1.44570	0.32731	-0.28905	-9.469
	A. nigrofasciatus	108	28	0.881±0.00045	0.00473±0.0000001	-1.72514	-3.19875*	-3.12552*	-13.945
	A. adloffi	2	2	1.000±0.25000	0.00289±0.0000021	-	-	-	-
	Austrolebias sp	5	5	1.000±0.01600	0.02338±0.0001283	-0.84029	-0.84029	-0.90790	0.271

N, number of individuals; h, number of haplotypes; Hd, haplotype diversity; sd, standard deviation; π, nucleotide diversity; Tajima's D, Fu and Li's D, Fu and Li's F, Fu's Fs, neutrality tests. Significant values ($p < 0.05$) are indicated by an "*".

Table 2 - Genetic diversity and neutrality tests performed for each taxonomic unity using the nuclear gene *rhodopsin*.

Molecular marker	Species	N	a	He/sd	π/sd	Tajima's D	Fu and Li's D	Fu and Li's F	Fu's Fs
Rhodopsin	*A. viarius*	10	5	0.867±0.00509	0.00229±0.0000001	0.08282	0.45041	0.40377	-1.356
	A. minuano + A. charrua	64	32	0.941±0.00026	0.00657±0.0000006	-1.31934	-0.78460	-1.17330	-21.576
	A. nachtigalli	40	19	0.919±0.00087	0.00540±0.0000002	-0.11200	0.69757	0.50895	-9.019
	A. reicherti	62	12	0.743±0.00288	0.00299±0.0000002	-0.75097	-1.93533	-1.81261	-3.485
	A. arachan	30	5	0.361±0.01194	0.00082±0.0000001	-1.60513	-0.60837	-1.04979	-2.391
	A. bagual	2	1	-	-	-	-	-	-
	A. pongondo	22	20	0.991±0.00027	0.01025±0.0000005	-0.82805	-0.84919	-0.98430	-13.004
	A. pelotapes	26	12	0.815±0.00524	0.00539±0.0000010	-1.26156	-1.04942	-1.30648	-3.174
	A. minuano affinis – SJN	12	9	0.955±0.00218	0.00443±0.0000003	-0.26201	0.54276	0.38063	-4.318
	A. minuano affinis – TAV	8	8	1.000±0.00391	0.01349±0.0000078	0.25831	0.90379	0.83774	-2.531
	A. nigrofasciatus	69	34	0.984±0.00002	0.00752±0.0000002	-0.16187	1.44144	1.01877	-21.064
	A. adloffi	6	6	1.000±0.00926	0.01069±0.0000053	0.60284	0.76035	0.78867	-1.606
	Austrolebias sp.	2	2	1.000±0.25000	0.00159±0.0000006	-	-	-	-

N, number of individuals; h, number of haplotypes; He, expected heterozigozity; sd, standard deviation; π, nucleotide diversity; Tajima's D, Fu and Li's D, Fu and Li's F, Fu's Fs, neutrality tests.

Table 3 - Pairwise FST values and pairwise mean distances obtained in the comparisons between the taxonomic unities using *cytb*. Significant values ($p < 0.05$) are indicated by an "*". Values above the diagonal refer to *FST* whereas those below the diagonal refer to pairwise mean distance.

	A. viarius	*A. minuano + A. charrua*	*A. nachtigalli*	*A. reicherti*	*A. arachan*	*A.bagual*	*A. pongondo*	*A. pelotapes*	*A. minuano affinis – SJN*	*A. minuano affinis – TAV*	*A. nigrofasciatus*	*A. adloffi*	*Austrolebias sp.*
A. viarius		0.95202*	0.84063*	0.94975*	0.96155*	0.94186*	0.96144*	0.94656*	0.86789*	0.95067*	0.95942*	0.90723*	0.85826*
A. minuano + A. charrua	0.111		0.58392*	0.93714*	0.95236*	0.96162*	0.96565*	0.96180*	0.90805*	0.95516*	0.96180*	0.95663*	0.93777*
A. nachtigalli	0.121	0.025		0.83845*	0.82592*	0.85760*	0.87044*	0.85674*	0.80085*	0.87367*	0.92971*	0.78916*	0.78188*
A. reicherti	0.124	0.066	0.071		0.94812*	0.95823*	0.96459*	0.95821*	0.90309*	0.95292*	0.96186*	0.95055*	0.92935*
A. arachan	0.136	0.067	0.075	0.071		0.97866*	0.98399*	0.97598*	0.90297*	0.96769*	0.96665*	0.98195*	0.94378*
A. bagual	0.113	0.098	0.106	0.104	0.114		0.96556*	0.95249*	0.80711*	0.93240*	0.94699*	0.94903*	0.80684*
A. pongondo	0.117	0.093	0.098	0.104	0.110	0.061		0.90494*	0.73663*	0.90521*	0.95176*	0.95011*	0.86461*
A. pelotapes	0.117	0.098	0.101	0.104	0.105	0.063	0.022		0.70023*	0.88216*	0.94468*	0.90004*	0.79672*
A. minuano affinis – SJN	0.114	0.090	0.092	0.094	0.104	0.053	0.031	0.032		0.32536*	0.90390*	0.31590	0.42203*
A. minuano affinis – TAV	0.117	0.090	0.093	0.095	0.108	0.054	0.031	0.030	0.013		0.78392*	0.92421*	0.51069*
A. nigrofasciatus	0.111	0.103	0.107	0.109	0.110	0.068	0.069	0.064	0.060	0.061		0.93866*	0.72142*
A. adloffi	0.118	0.089	0.090	0.00.094	0.103	0.056	0.031	0.028	0.017	0.013	0.064		0.94327*
Austrolebias sp.	0.108	0.091	0.093	0.094	0.105	0.038	0.037	0.034	0.026	0.029	0.060	0.028	

Table 4 - Pairwise FST values and pairwise mean distances obtained in the comparisons between the taxonomic unities using *rhodopsin*. Significant values ($p < 0.05$) are indicated by an "*". Values above the diagonal refer to *FST* whereas those below the diagonal refer to pairwise mean distance.

	A. viarius	*A. minuano + A. charrua*	*A. nachtigalli*	*A. reicherti*	*A. arachan*	*A.bagual*	*A. pongondo*	*A. pelotapes*	*A. minuano affinis – SJN*	*A. minuano affinis – TAV*	*A. nigrofasciatus*	*A. adloffi*	*Austrolebias sp.*
A. viarius		0.27973*	0.44880*	0.69687*	0.87597*	0.56081*	0.15646*	0.47018*	0.44801*	0.39605*	0.27143*	0.55752*	0.57317*
A. minuano + A. charrua	0.000		0.21554*	0.48382*	0.55528*	0.29920*	0.20714*	0.35154*	0.38679*	0.41791*	0.32888*	0.52886*	0.31446*
A. nachtigalli	0.000	0.000		0.33419*	0.59011*	0.37263*	0.17035*	0.34470*	0.36450*	0.37520*	0.30261*	0.51161*	0.40645*
A. reicherti	0.000	0.000	0.000		0.69639*	0.63863*	0.38119*	0.50788*	0.56783*	0.56630*	0.42580*	0.67359*	0.65731*
A. arachan	0.000	0.000	0.000	0.000		0.90312*	0.47067*	0.68262*	0.75341*	0.65406*	0.49288*	0.79844*	0.90188*
A. bagual	0.000	0.000	0.000	0.000	0.000		0.01640	0.40860*	0.36625*	0.08681	0.08819	0.24533	0.66667
A. pongondo	0.001	0.001	0.001	0.001	0.001	0.001		0.15051*	0.07244*	0.13101*	0.12691*	0.22535*	0.00377
A. pelotapes	0.000	0.000	0.000	0.000	0.000	0.000	0.001		0.14856*	0.26131*	0.32361*	0.37520*	0.40672*
A. minuano affinis – SJN	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000		0.12427*	0.27255*	0.28924*	0.39216*
A. minuano affinis – TAV	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004		0.10825	0.20644*	0.25091
A. nigrofasciatus	0.000	0.001	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.004		0.33542*	0.17029*
A. adloffi	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.007	0.000		0.40842*
Austrolebias sp.	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.004	0.000	0.004	

Table 5 - Pairwise FST values and pairwise mean distances obtained in the comparisons between the taxonomic unities using *COI*. Significant values ($p < 0.05$) are indicated by an "*". Values above the diagonal refer to *FST* whereas those below the diagonal refer to pairwise mean distance.

	A. viarius	*A. minuano + A. charrua*	*A. nachtigalli*	*A. reicherti*	*A. arachan*	*A. bagual*	*A. pongondo*	*A. pelotapes*	*A. minuano affinis – SJN*	*A. minuano affinis – TAV*	*A. nigrofasciatus*	*Austrolebias sp.*
A. viarius		0.95097*	0.94361*	0.92749*	0.99617*	0.98909*	0.99892*	0.99686*	0.93811*	0.98687*	0.97162*	0.99774*
A. minuano + A. charrua	0.112		0.63546*	0.89869*	0.92215*	0.95411*	0.95394*	0.95697*	0.91899*	0.95774*	0.95506*	0.94221*
A. nachtigalli	0.113	0.019		0.89001*	0.90804*	0.94702*	0.94848*	0.95230*	0.91574*	0.95462*	0.95408*	0.93355*
A. reicherti	0.114	0.072	0.073		0.91377*	0.93920*	0.93885*	0.94362*	0.92135*	0.95019*	0.95543*	0.92576*
A. arachan	0.110	0.057	0.056	0.078		0.99004*	0.99838*	0.99654*	0.92410*	0.98471*	0.96805*	0.99741*
A. bagual	0.116	0.098	0.095	0.110	0.097		0.98805*	0.98710*	0.89483*	0.97472*	0.94834*	0.98254*
A. pongondo	0.122	0.092	0.091	0.109	0.090	0.056		0.98230*	0.80661*	0.95683*	0.94502*	1.00000*
A. pelotapes	0.120	0.095	0.094	0.112	0.093	0.059	0.009		0.82521*	0.96149*	0.95004*	0.99272*
A. minuano affinis – SJN	0.119	0.087	0.086	0.109	0.085	0.056	0.119	0.031		0.59387*	0.91432*	0.59604*
A. minuano affinis – TAV	0.117	0.086	0.086	0.104	0.086	0.056	0.117	0.029	0.012		0.92970*	0.94489*
A. nigrofasciatus	0.118	0.088	0.090	0.108	0.097	0.058	0.118	0.057	0.055	0.053		0.94489*
Austrolebias sp.	0.125	0.090	0.088	0.110	0.091	0.057	0.125	0.032	0.014	0.018	0.056	

FIGURES

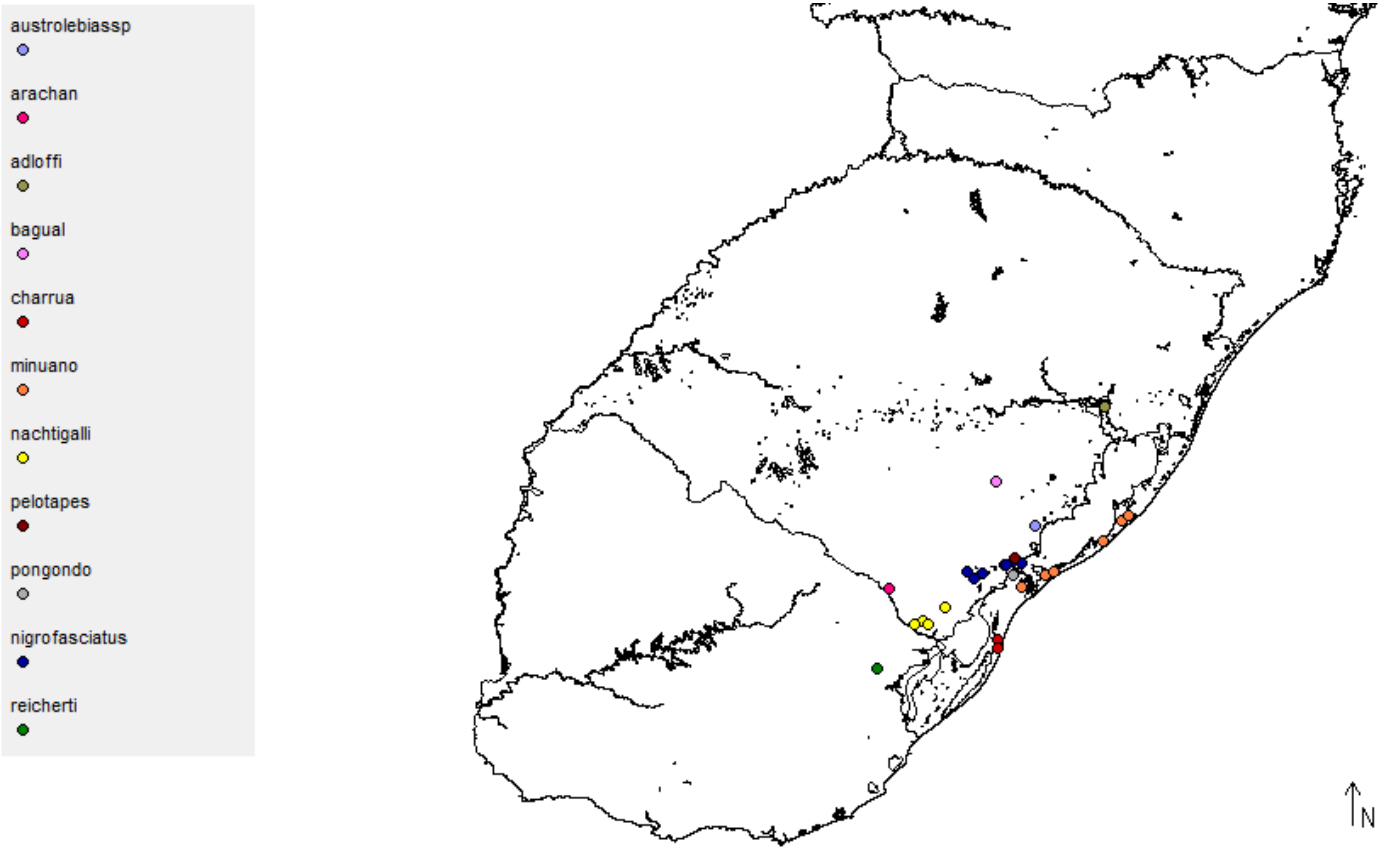

Figure 1 – Map showing the collected points of the species of *A. adloffi* species group. Dots refer to populations. Colors refer to species, indicated on legend at left.

Figure 2 – Phylogenetic tree obtained by Bayesian Analisys using *COI*.

Figure 3 – Phylogenetic tree obtained by Bayesian Analisys using *cytb*.

Figure 4 – Phylogenetic tree obtained by Bayesian Analisys using concatenated mitochondrial sequences.

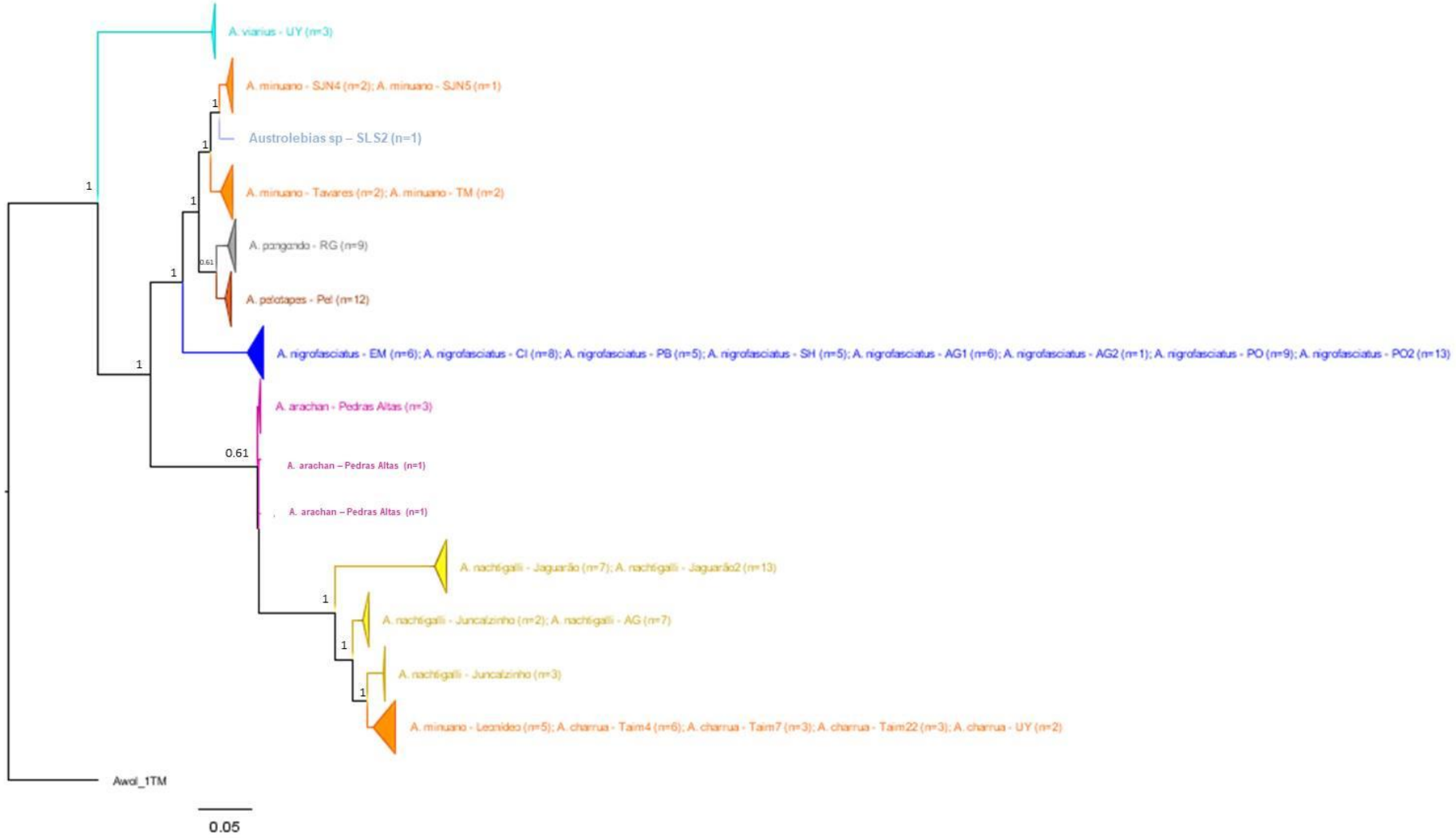

Figure 5 – Phylogenetic tree obtained by Bayesian Analisys using concatenated sequences of *COI*, *cytb* and *rhodopsin*.

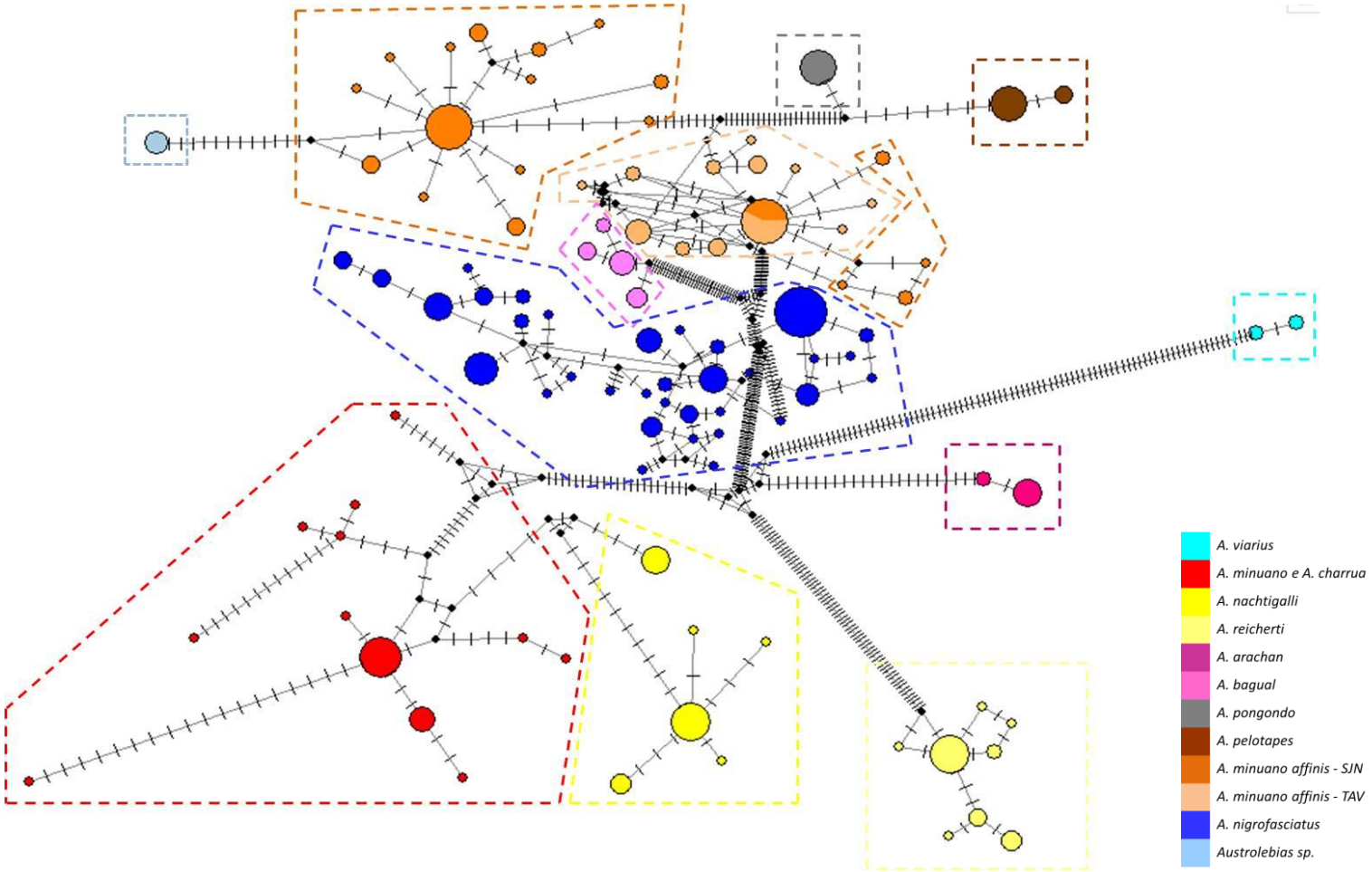

Figure 6 – Median-joining network of the 350 *COI* sequences (931 pb) sampled for the *A. adloffi* species group. The size of circles is proportional to the haplotype frequency. The colors refer to taxonomic unities, given in the legend to the right.

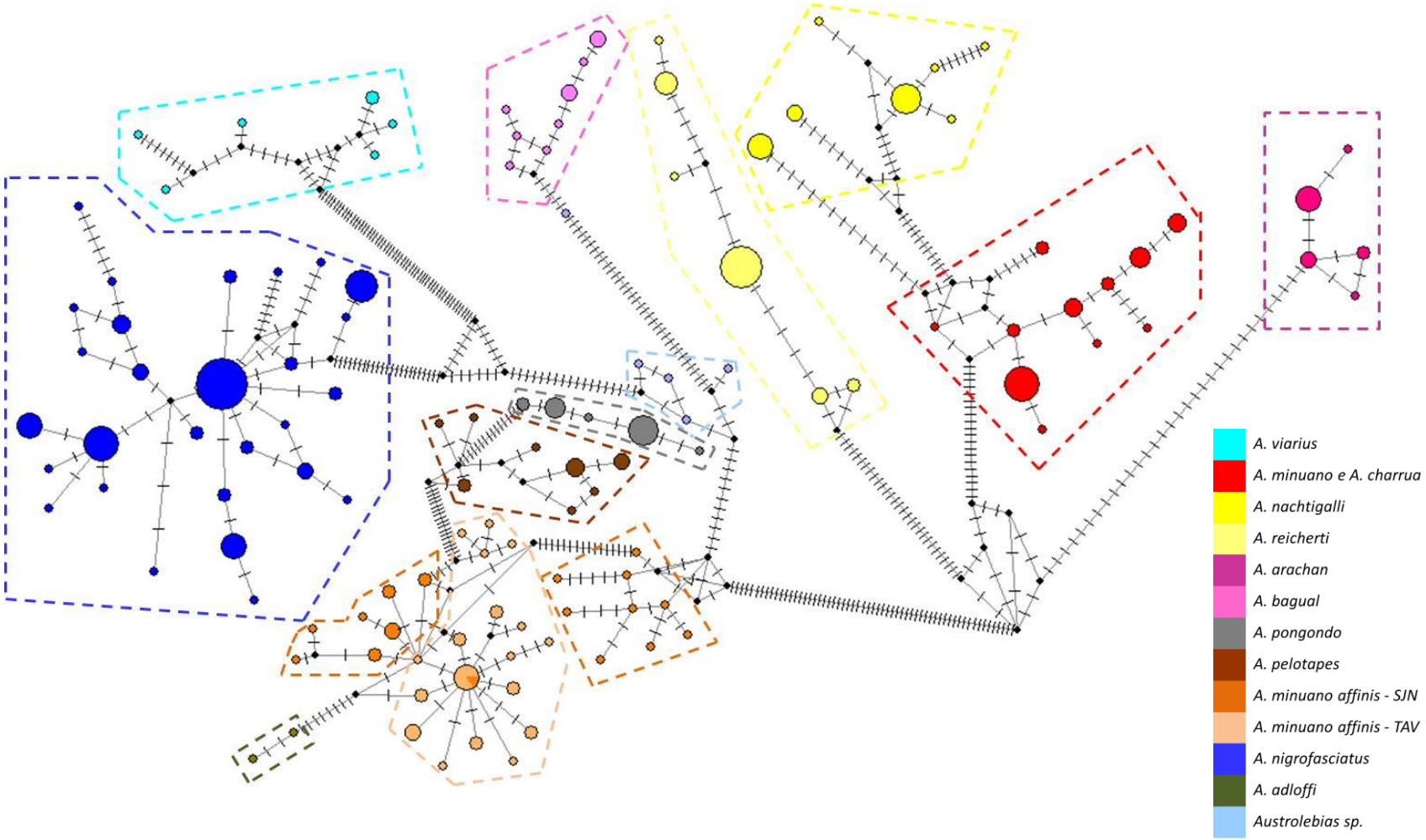

Figure 7 – Median-joining network of the 332 *cytb* sequences (693 pb) sampled for the *A. adloffi* species group. The size of circles is proportional to the haplotype frequency. The colors refer to taxonomic unities, given in the legend to the right.

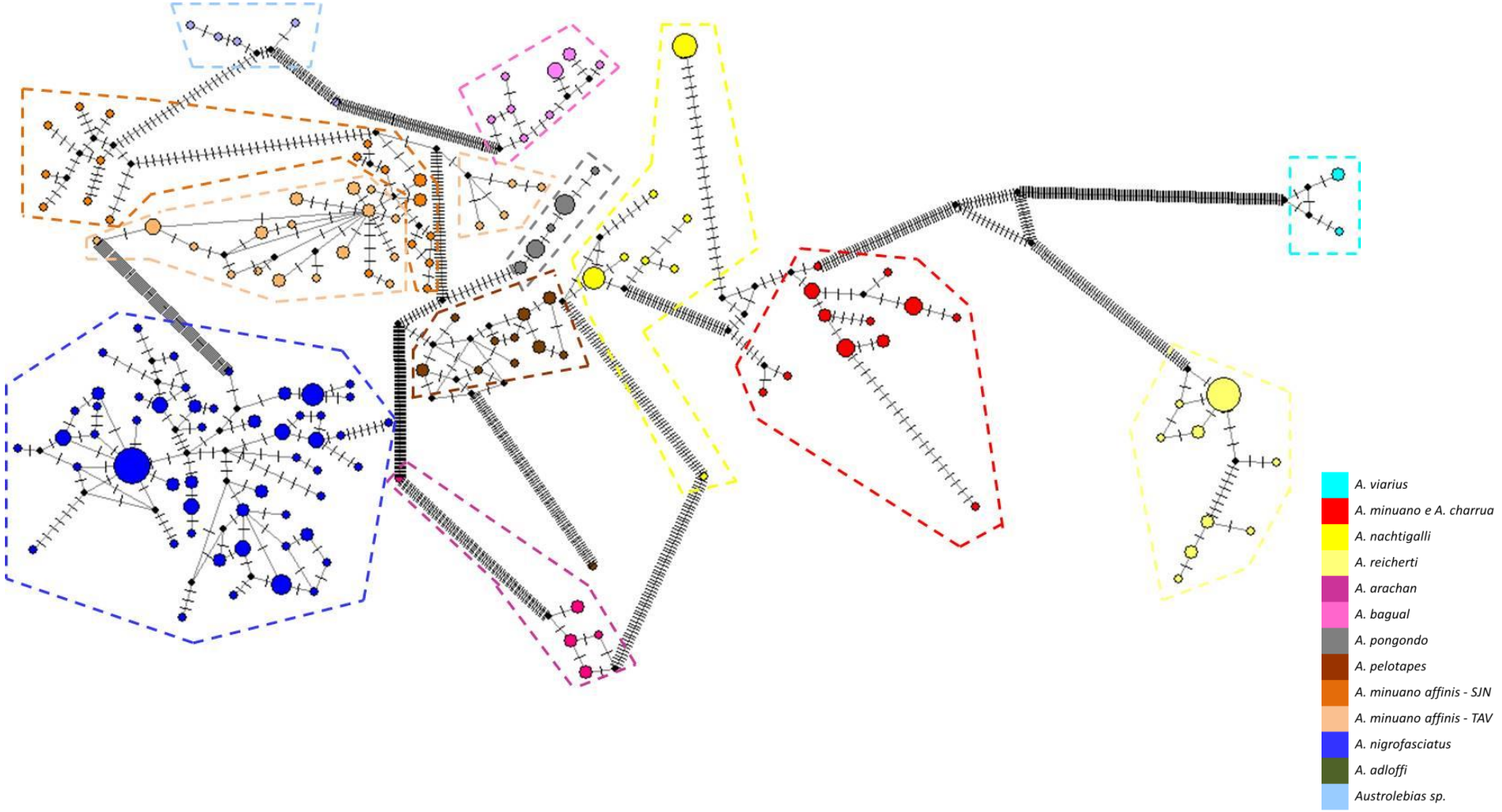

Figure 8 – Median-joining network of the 267 concatenated mitochondrial sequences (1624pb) sampled for the *A. adloffi* species group. The size of circles is proportional to the haplotype frequency. The colors refer to taxonomic unities, given in the legend to the right.

SUPPLEMENTARY MATERIAL

Table S1 – Number of individuals collected for each population of the 11 species of *A. adloffi* species group. n= number of individuals.

Species	Population	n	Coordinates
A. arachan	Pedras Altas	20	53W 43' 10", 32S 03' 42"
A. varius	Uruguay	5	
A. adloffi	Eldorado do Sul	2	51W 16' 04", 30S 00' 11"
A. pelotapes	Pelotas	16	52W 18' 03", 31S 42' 48"
A. pongondo	Povo Novo – RG	20	52W 18' 47", 31S 55' 21"
A. bagual	Encruzilhada do Sul	18	52º30'10.8'', 30º51'21.8"
A. nigrofasciatus	Arroio Grande – 1	11	52W 39' 24", 31S 53' 33"
	Arroio Grande – 2	3	
	Capão do Leão	21	52W 23' 41", 31S 47' 47"
	Av. Eliseu Maciel	20	52W 25' 09", 31S 48' 19"
	Pontal da Barra	14	52W 13' 53", 31S 46' 42"
	Shopping Pelotas	17	52W 18' 52", 31S 45' 45"
	Pedro Osório – 1	11	52W 45' 36", 31S 56' 46"
	Pedro Osório – 2	20	52W 50' 10", 31S 52' 15"
A. nachtigalli	Arroio Grande	20	53W 05' 52", 32S 16' 18"
	Arroio Juncalzinho	15	53W 17' 11", 32S 28' 00"
	Jaguarão – 1	11	53W 26' 42", 32S 27' 49"
	Jaguarão – 2	19	53W 20' 26", 32S 26' 18"
A. reicherti	Uruguay	5	53W54'49'', 32S55'21''
A. charrua	Taim4	9	32º39'15.3'', 52º29'16.6"
	Taim7	3	32º38'20.5'', 52º29'15.9"
	Taim22	16	32º44'52.8'', 52º29'41.9"
	Uruguay	10	
A. minuano	Leonídeo	18	52W 13' 41", 32S 03' 02"
	São José do Norte – 3	18	51W 56' 35", 31S 55' 02"
	São José do Norte – 4	17	51W 51' 43", 31S 52' 49"
	São José do Norte – 5	18	51W 17' 28", 31S 31' 38"
	Tavares	20	51W 04' 53", 31S 17' 30"
	Tavares – TM	19	51W 00' 18", 31S 14' 27"

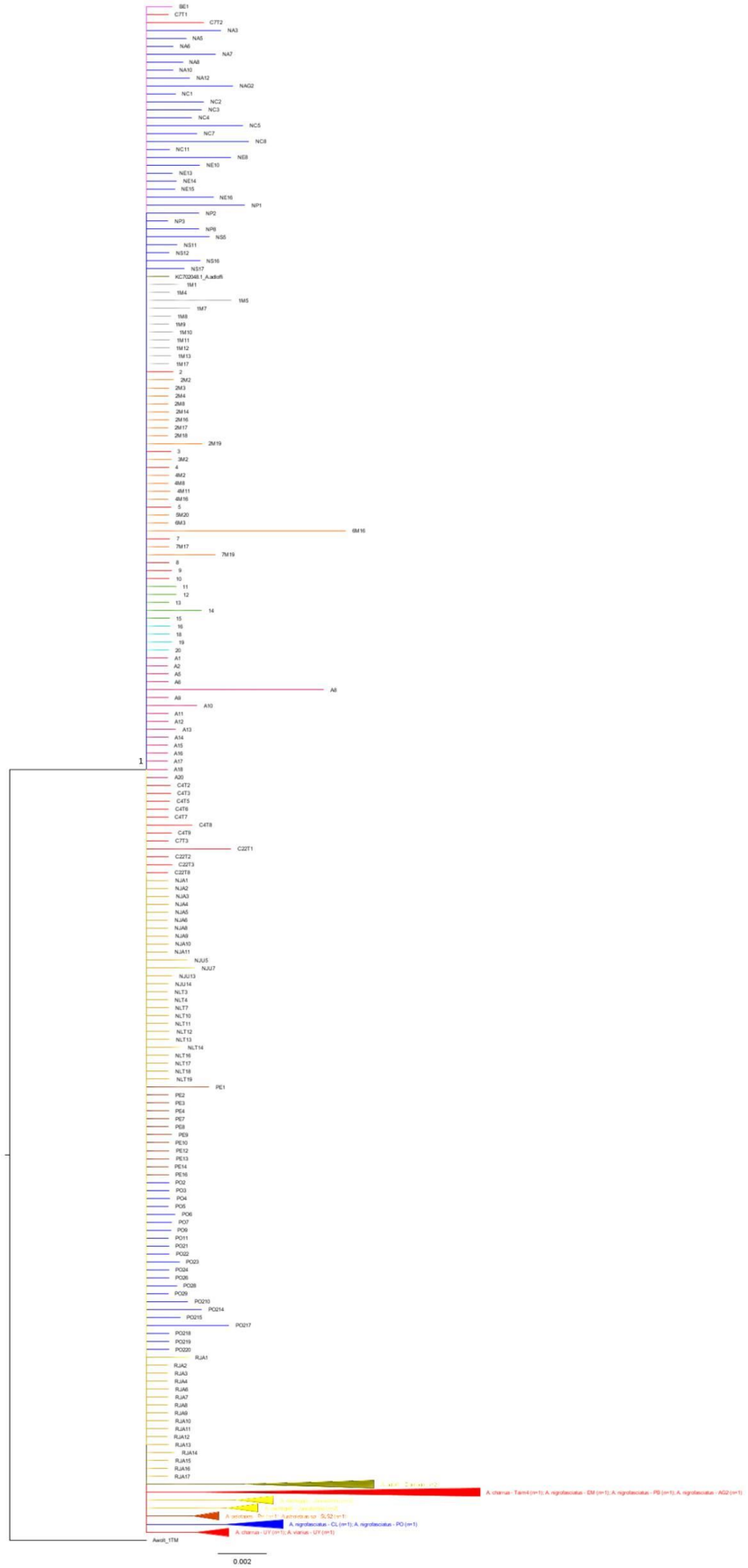

Figure S1 – Phylogenetic tree obtained by Bayesian Analisys using *rhodopsin.*

PCR Conditions:

For *COI* and *cytb*: 20 µL total volume using 2 µL of 10X buffer, 1 µL of $MgCl_2$ (50 mM), 2.5 µL of dNTPs (2 mM), 0.3 µL of each primer (20 µM), 0.2 µL of *Taq* DNA polymerase (5 U/µL) and 2 µL of DNA. For cytb was added5% of DMSO. Both the markers under the following conditions: 94 ºC for 5 min, 35 cycles of 40 s at 94 ºC, 40 s at 48 ºC; 1 min at 72 ºC, and a final extension step of 10 min at 72 ºC.

For *rhodopsin*: 20 µL total volume using 2 µL of 10X buffer, 1 µL of $MgCl_2$ (50 mM), 2.5 µL of dNTPs (2 mM), 0.5 µL of each primer (20 µM), 0.3 µL of *Taq* DNA polymerase (5 U/µL) and 3 µL of DNA. Under the following conditions: 94 ºC for 4 min, 30 cycles of 30 s at 94 ºC, 30 s at 54 ºC; 30 s at 72 ºC, and a final extension step of 4 min at 72 ºC.

INSTRUÇÕES AOS AUTORES

JOURNAL OF ZOOLOGICAL SYSTEMATICS AND EVOLUTIONARY RESEARCH

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(Page break)

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(Page break)

Main body text (see Section 3 for structure); Acknowledgements.

(Page break)

Second Summary (if available)

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References

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Figure legends; List of Supporting Information (if available; only headers of figures and tables)

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Tables (consecutively numbered; each table complete with title and footnotes)

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Appendices (if available).

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Trade Names: Chemical substances should be referred to by the generic name only. Trade names should not be used. Drugs should be referred to by their generic names. If proprietary drugs have been used in the study, refer to these by their generic name, mentioning the proprietary name and the name and location of the manufacturer in parentheses.

Where commercially available substances, reagents, or equipment are used, the manufacturer's name and address (city and country is sufficient) should be provided in the 'Materials and Methods' section, along with the generally accepted common name.

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References should be prepared according to the Publication Manual of the American Psychological Association (6th edition). This means in text citations should follow the author-date method whereby the author's last name and the year of publication for the source should appear in the text, for example, (Jones, 1998). The use of et al is determined by the number of authors and whether it is the first time a reference has been cited in the paper:

- articles with one or two authors include all names in every in-text citation;
- articles with three, four, or five authors include all names in the first in-text citation but are abbreviated to the first author name plus et al. upon subsequent citations;
- articles with six or more authors are abbreviated to the first author name plus et al. for all in-text citations.

The complete reference list should appear alphabetically by name at the end of the paper. A sample of the most common entries in reference lists appears below. Please note that a DOI should be provided for all references where available. For more information about APA referencing style, please refer to the References FAQ . Please note that for journal articles, issue numbers are not included unless each issue in the volume begins with page one.

Journal article

Beers, S. R. , & De Bellis, M. D. (2002). Neuropsychological function in children with maltreatment-related posttraumatic stress disorder. *The American Journal of Psychiatry*, 159, 483–486. doi:10.1176/appi.ajp.159.3.483

Book

Bradley-Johnson, S. (1994). *Psychoeducational assessment of students who are visually impaired or blind: Infancy through high school* (2nd ed.). Austin, TX: Pro-ed.

Chapter in an Edited Book

Borstrøm, I., & Elbro, C. (1997). Prevention of dyslexia in kindergarten: Effects of phoneme awareness training with children of dyslexic parents. In C. Hulme & M. Snowling (Eds.), *Dyslexia: Biology, cognition and intervention* (pp. 235–253). London: Whurr.

Internet Document

Norton, R. (2006, November 4). *How to train a cat to operate a light switch* [Video file]. Retrieved from http://www.youtube.com/watch?v=Vja83KLQXZs

4.4.3. Figures and Tables

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Note: if data, scripts, or other artefacts used to generate the analyses presented in the paper are available via a publicly available data repository, authors should include a reference to the location of the material within their paper.

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Author Guidelines updated November 2018

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

O grupo de espécies *Austrolebias adloffi* conta, até o momento, com 11 espécies válidas descritas. O presente estudo, porém, revelou que este grupo apresenta 13 unidades taxonômicas monofiléticas, as quais poderiam, eventualmente, constituir 13 espécie distintas. No entanto, não se trata de apenas duas espécies além do inicial, mas de modificações em toda a taxonomia do grupo. Ao passo que *A. nachtigalli* seria subdividida em duas, uma destas incluiria *A. reicherti*; enquanto *A. minuano* se subdivide em três clados distintos, um destes se mistura a *A. charrua*. Por sua vez, *A. nigrofasciatus*, a qual acreditava-se ter apenas 5 populações, mostrou ter uma distribuição maior, contando com 8 populações.

Tais modificações têm influência direta sobre a real área de distribuição das espécies e, consequentemente, no status de ameaça de extinção das mesmas. Deste modo, se tornam necessários estudos mais detalhados acerca da validação destas 13 unidades taxonômicas como sendo 13 espécies, visando a elucidação de questões filogenéticas e também a preservação destas espécies já extremamente ameaçadas.

Neste sentido, este trabalho abre diversas perspectivas futuras, tais como:

- aumento do n amostral para algumas espécies pouco amostradas e cujas questões taxonômicas permaneceram com questionamentos (*A. adloffi*, *A. nachtigalli*, *A. reicherti*, *A. arachan*);

-amostragem incluindo a espécie *A. araucarianus*, a se mostrou pertencente ao grupo *A. adloffi* em estudos anteriores;

- realização de análises mais refinadas a fim de confirmar a monofilia recíproca das unidades taxonômicas;

- realização de análises cronofilogenéticas, possibilitando relacionar a diversificação do grupo com a formação geográfica da região em que vivem;

- realização de estudos morfométricos, a fim de avaliar o grau de distinção morfológica entre os diferentes agrupamentos.

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CEUA

CERTIFICADO Nº P045/2016

Certificamos que o projeto intitulado "Taxonomia molecular, diversidade e estruturação genética de peixes anuais no Rio Grande do Sul (Cyprinodontiformes: Rivulidae)", protocolo nº 23116.008163/2015-23, sob a responsabilidade de Lizandra Jaqueline Robe - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi APROVADO pela COMISSÃO DE ÉTICA EM USO ANIMAL DA UNIVERSIDADE FEDERAL DO RIO GRANDE (CEUA-FURG), em reunião de 25 de maio de 2016 (Ata 006/2016).

A CEUA lembra aos pesquisadores que qualquer alteração no protocolo experimental ou na equipe deve ser encaminhada à comissão para avaliação e aprovação. Um relatório final deve ser enviado à CEUA no término da vigência do seu projeto.

CEUA Nº	Pq036/2015
COLABORADORES	Daniel Loebmann; Daiana Kaster Garcez; Matheus Vieira Volcan; CrislaineBarbosa.
VIGÊNCIA DO PROJETO	31/03/2020
ESPÉCIE/ LINHAGEM	*Austrolebias sp.*, *A. minuano*, *A. nigrofasciatus*, *A. bagual*, *Cynopoecilus melanotaenia*, *C. intimus*, *C. nigrovittatus*, *C. notabilis*.
NÚMERO DE ANIMAIS	20 indivíduos de cada população.
PESO/ IDADE	1 g / Adulto
SEXO	Indiferente
ORIGEM	Sistemas de drenagens Patos-Mirim e áreas costeiras adjacentes.
ENVIO DO RELATÓRIO FINAL	Abril de 2020.

Rio Grande, 08 de junho de 2016.

Med. Vet. Márcio de Azevedo Figueiredo
Coordenador da CEUA-FURG

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AUTORIZAÇÃO DE ALTERAÇÃO DE PROTOCOLO EXPERIMENTAL Nº 007/2017

PROCESSO Nº	23116.008163/2015-23
CEUA Nº	Pq036/2015
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 / 31/03/2020
ENVIO DO RELATÓRIO FINAL	Abril de 2020
PROFESSOR RESPONSÁVEL	Lizandra Jaqueline Robe

PARECER DA CEUA:

Após a análise de sua solicitação de alteração de protocolo experimental, enviada a essa comissão em 16 de maio de 2017, a CEUA-FURG **AUTORIZA** a inclusão das seguintes espécies no projeto: *Cynopoecilus* sp., *C. fulgens*, *C. multipapillatus*, *C. feltrini*, *Austrolebias wolterstorffi*, *A. charrua*, *A.adloffi*, *A. arachan*, *A. viarius*, *A. nachtigalli* e *A. reicherti*, sendo mantidas exatamente as mesmas condições experimentais aprovadas pela CEUA-FURG.

Rio Grande, 31 de maio de 2017.

Med. Vet. Márcio de Azevedo Figueiredo
Coordenador da CEUA-FURG

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 55651-1	Data da Emissão: 20/10/2016 09:57	Data para Revalidação*: 19/11/2017

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Dados do titular

Nome: Daiana Kaster Garcez	CPF: 020.671.830-60
Título do Projeto: Taxonomia molecular, diversidade e estruturação genética de peixes anuais da planície costeira do Rio Grande do Sul, Brasil (Cyprinodontiformes: Rivulidae)	
Nome da Instituição : UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG	CNPJ: 94.877.586/0001-10

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta dos indivíduos	10/2016	10/2017
2	Revisão bibliográfica	10/2016	01/2020
3	Extração de DNA	10/2016	02/2018
4	Amplificação, purificação e sequenciamento	10/2016	12/2018
5	Análise de dados	01/2017	06/2019
6	Entrega e defesa da dissertação	02/2020	03/2020

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#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	Crislaine Barbosa	Coleta de dados	039.428.190-07	8118540486 SSP-RS	Brasileira
2	Lizandra Jaqueline Robe	Pesquisadora associada	969.799.840-04	1057032847 SSP-RS	Brasileira

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Nome da Instituição : UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG	CNPJ: 94.877.586/0001-10

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Número: 55651-1	Data da Emissão: 20/10/2016 09:57	Data para Revalidação*: 19/11/2017

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Dados do titular

Nome: Daiana Kaster Garcez	CPF: 020.671.830-60
Título do Projeto: Taxonomia molecular, diversidade e estruturação genética de peixes anuais da planície costeira do Rio Grande do Sul, Brasil (Cyprinodontiformes: Rivulidae)	
Nome da Instituição : UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG	CNPJ: 94.877.586/0001-10

46	GRAVATAI	RS	Diversas	Fora de UC Federal
47	CAPAO DA CANOA	RS	Diversas	Fora de UC Federal
48	XANGRI-LA	RS	Diversas	Fora de UC Federal
49	OSORIO	RS	Diversas	Fora de UC Federal
50	IMBE	RS	Diversas	Fora de UC Federal
51	TRAMANDAI	RS	Diversas	Fora de UC Federal
52	CIDREIRA	RS	Diversas	Fora de UC Federal
53	CAPIVARI DO SUL	RS	Diversas	Fora de UC Federal
54	PALMARES DO SUL	RS	Diversas	Fora de UC Federal
55	MOSTARDAS	RS	Diversas	Fora de UC Federal
56	TAVARES	RS	Diversas	Fora de UC Federal
57	SAO JOSE DO NORTE	RS	Diversas	Fora de UC Federal
58	ARROIO DO SAL	RS	Diversas	Fora de UC Federal
59	TORRES	RS	Diversas	Fora de UC Federal
60	PASSOS DE TORRES	SC	Diversas	Fora de UC Federal
61	SAO JOAO DO SUL	SC	Diversas	Fora de UC Federal
62	SANTA ROSA DO SUL	SC	Diversas	Fora de UC Federal
63	SOMBRIO	SC	Diversas	Fora de UC Federal
64	ARARANGUA	SC	Diversas	Fora de UC Federal
65	ICARA	SC	Diversas	Fora de UC Federal
66	SANGAO	SC	Diversas	Fora de UC Federal
67	JAQUIRANA	RS	Diversas	Fora de UC Federal
68	TUBARAO	SC	Diversas	Fora de UC Federal
69	CAPIVARI DE BAIXO	SC	Diversas	Fora de UC Federal
70	LAGUNA	SC	Diversas	Fora de UC Federal

Atividades X Táxons

#	Atividade	Táxons
1	Coleta/transporte de amostras biológicas in situ	Rivulidae
2	Coleta/transporte de espécimes da fauna silvestre in situ	Rivulidae (*Qtde: 20)

* Quantidade de indivíduos por espécie, por localidade ou unidade de conservação, a serem coletados durante um ano.

Material e métodos

1	Amostras biológicas (Peixes)	Fragmento de tecido/órgão
2	Método de captura/coleta (Peixes)	Picaré, Armadilha (covo, manzuá, potes para polvos, substrato específico, manilha e variações), Puçá

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	UFRGS - UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL	coleção

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Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

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* Identificar o espécime no nível taxonômico possível.

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Phylogeography of the critically endangered neotropical annual fish, *Austrolebias wolterstorffi* (Cyprinodontiformes: Aplocheilidae): genetic and morphometric evidence of a new species complex

Daiana K. Garcez · Crislaine Barbosa · Marcelo Loureiro · Matheus V. Volcan · Daniel Loebmann · Fernando M. Quintela · Lizandra J. Robe

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Abstract *Austrolebias wolterstorffi* is a critically endangered annual fish, occurring in temporary ponds in a restricted area of Southern Brazil and Uruguay. Here, we evaluate the levels of genetic diversity and morphometric differentiation presented by *A. wolterstorffi*, attempting to reconstruct the spatiotemporal scenario by which this species reached their current distribution. Part of the mitochondrial cytochrome *b* and nuclear rhodopsin genes were characterized and analysed for a set of 122 and 110 specimens, respectively, collected along the entire distribution range of the species. Additionally, shape variations were evaluated for 92 individuals (43 males and 49 females) through geometric morphometric methods. Our analyses demonstrated several cases of significantly high levels of genetic differentiation among individual populations, in an isolation-by-distance pattern of divergence, with at least six different population groups along the Patos-Mirim lagoon. These groups differed by a minimum of 0.9% and a maximum of 2.6% of corrected cyt *b* nucleotide distances and did not share any mitochondrial haplotype. Such a pattern, added to the slight morphometric differentiation detected for most of the groups, suggests the occurrence of incipient speciation as consequence of allopatric fragmentation. The chronophylogenetic tree performed with the concatenated dataset supported independent oriental and occidental colonization routes, with the population located in the northwest part of the Rio Grande do Sul coastal plain presenting the most ancient divergence. In general, the recovered biogeographic patterns are highly consistent with the records of Quaternary climatic changes and depositional events that have occurred along the area inhabited by the studied species. This allowed us to establish a molecular clock calibration system for Neotropical annual fish. Thus, although the taxonomic status of each of the detected population units needs further study, it is clear that independent conservation strategies must be taken in each of the major areas covered by this study, most of which are located in Brazil.

Keywords Allopatric fragmentation · Cryptic speciation · Genetic structure · Patos-Mirim lagoon system

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Introduction

Freshwater annual fish (Cyprinodontiformes: Aplocheiloidei) possess uncommon developmental, ecological, and physiological adaptations. They have an unusually short life cycle of less than one year, which is entirely correlated to the seasonal ponds they inhabit (Loureiro and de Sá 2015). Their non-overlapping generation times and habitats make annual fish an excellent model for studies of evolution. Also, their patchy distribution provides ideal conditions for studying the underlying evolutionary mechanisms (e.g., genetic drift, gene flow, selection) that usually result in rapid divergence between populations or even allopatric speciation with or without morphological differentiation (de Sá et al. 2015).

Among Aplocheiloidei, Aplocheilidae is one of the most diverse families, with about 350 described species living in South and Central America and in the southern United States (Costa 2008). *Austrolebias* currently encompasses 45 annual species, distributed along the Paraná-La Plata, Amazonas and Patos-Mirim basins (Costa 2006; Nielsen and Pillet 2015). This genus is particularly diverse in southern Brazil and Uruguay, especially in the Patos-Mirim lagoon system (Costa 2006). Nevertheless, several of these species are endangered because of their frequently restricted and patchy distributions, their characteristic low vagility, and the loss and fragmentation of their habitats (ICMBio 2013; Volcan et al. 2015).

Austrolebias wolterstorffi is one of the largest species of annual fish and has one of the widest distribution ranges within the genus (~50,000 km^2), occurring in temporary ponds from the north of the Patos Lagoon to the south of the Mirim Lagoon, along Southern Brazil and in Uruguay (Loureiro et al. 2015). It is considered a critically endangered (CR) species (Reis et al. 2003; ICMBio 2013) because of a combination of its peculiar evolutionary properties and the loss and degradation of its habitats. Nevertheless, despite the incipient incentive of studies aiming to enhance the knowledge of annual fish (ICMBio 2013), even the phylogenetic position of *A. wolterstorffi* remains controversial. In this sense, although classical studies (Costa 2006, 2010) tend to allocate this species as a member of the *A. elongatus* group, a recent analysis (García et al. 2014) recovered it as an early offshoot within its genus.

Although the relatively wider geographical distribution of *A. wolterstorffi* suggests it might recover more easily than other congeners annual fish, the combination of high levels of genetic drift, frequent bottlenecks, inbreeding, and low levels of gene flow (de Sá et al. 2015) might have led to population differentiation or unrecognized cryptic speciation, which could affect its long-term persistence. Therefore, we aimed to assess the levels of diversity and the genetic and morphometric structure within and among populations of *A. wolterstorffi*, attempting to help in the reconstruction of its evolutionary history and in the establishment of management and conservation strategies.

Materials and methods

Study area

This study includes molecular and/or morphometric data from a total of 134 individuals of *A. wolterstorffi* collected between 2014 and 2015 in 22 sampling locations distributed in the entire known distribution range of the species (Loureiro et al. 2015; Volcan et al. 2015), which comprises the Patos-Mirim lagoon system, in the southernmost Brazilian state of Rio Grande do Sul and Uruguay (Fig. 1; Supporting Information Table 1S and 2S).

With the exception of Eldorado do Sul, all of the sampled sites in the Rio Grande do Sul Brazilian state are within Quaternary sedimentary deposits of the coastal plain. This region was intensely reworked during the paleoclimatic alternations of Quaternary, which caused variations in sea level, thereby opening and closing areas of communication with the Atlantic Ocean, and building a system referred to as the Multiple Barrier (Villwock and Tomazelli 2007). This system is formed by four major depositional events (Barrier I–IV) that extended from 400 to 5 thousand years ago (kyr) and led to the formation of the Patos, Mirim, and Mangueira lagoons. The locality of Eldorado do Sul is inserted in the western portion of the Permian-Triassic sedimentary deposits, locally named the Peripheral Depression, which is part of the Paraná geological basin. Sampled localities in Uruguay are distributed along the Precambrian Shield, Paraná basin (Permian sedimentary and Mesozoic volcanic formation), and Quaternary sedimentary deposits (Rocha department) (Bossi and Navarro 1988).

Samplings

The fishes were collected in temporary ponds with the help of hand nets, euthanized with an overdose of

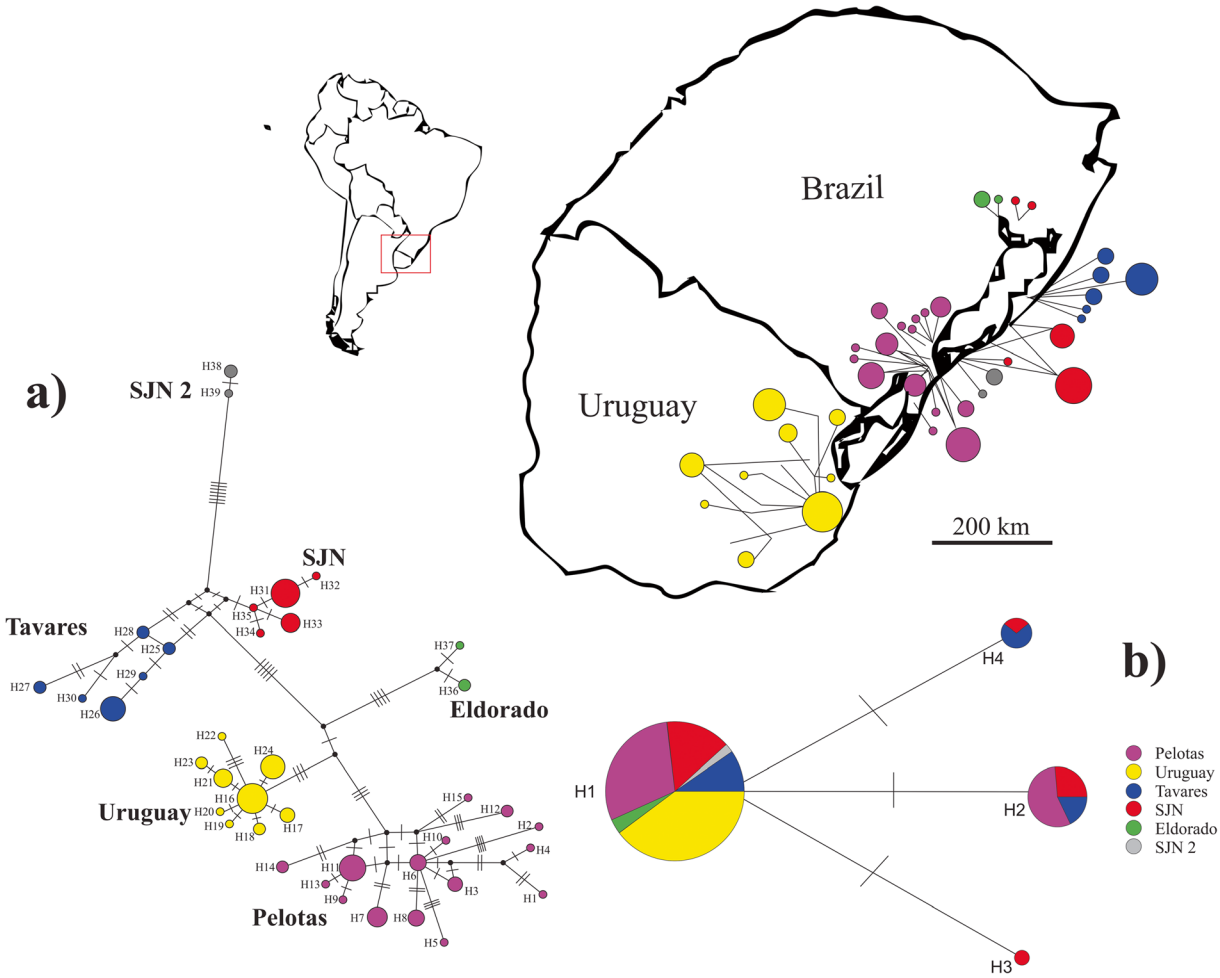

Fig. 1 Median-joining networks of the 39 cyt *b* haplotypes (**a**) and of the four RHO alleles (**b**). The size of each circle is proportional to haplotype frequencies, and colours refer to the main population groups recovered in this study (see Results) in relation to their geographic distribution (presented in the map)

3000 mg/L of eugenol anesthetic and then fixed in 95% ethanol. For the molecular procedures, a piece of the caudal peduncle was dissected from a set of 83 and 39 Brazilian and Uruguayan specimens, respectively (Table 2S). For geometric morphometry, 43 males and 49 females were photographed on their left side with a digital câmera (Olympus VG-120, supermacro mode).

DNA manipulation

Total DNA was extracted from each individual from approximately 30 mg of muscular tissue, using a phenol/chloroform protocol (Sambrook et al. 1989). Approximately 800 bp of the mitochondrial cytochrome *b* (cyt *b*) gene and 820 bp of the nuclear rhodopsin (RHO) were amplified from each sample using the primers L14735 (5′-AAAAACCACCGTTGTTATTCAACTA-3′) and CB3-H (5′-GGCAAATAGGAARTATCATTC-3′) (Palumbi et al. 1991; Wolf et al. 1999) and Rh193 (5′-CNTATGAATAYCCTCAGTACTACC-3′) and Rh1039r (5′-TGCTTGTTCATGCAGATGTAGA-3′) (Chen et al. 2003), respectively. PCR reactions were carried out using 100 ng of DNA in 25 μL reactions, containing 1× buffer, 0.5–1 μM of each primer, 0.25 mM of each dNTP, 2.5–3 mM of $MgCl_2$ and 1–1.5 U of Taq DNA polymerase, with the aid of 5% Dimethyl Sulfoxide in the amplification of RHO. PCR conditions for cyt *b* consisted of an initial stage of denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 60 s, and a final extension stage at 72 °C for 10 min; for RHO, cycling consisted of denaturation at 94 °C for

30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. To check whether the amplification was successful, 5 μL of the PCR product were separated by agarose (0.8%) gel electrophoresis and stained with GelRed (Biotium). The amplified fragments were then purified with a solution of 7.5 M ammonium acetate ($C_2H_7NO_2$) and directly sequenced. Sequencing was performed in a Perkin-Elmer ABI Prism 377 Automated Sequencer (MACROGEN, Seoul, Korea) using the same amplification primers.

Data analysis

First, electropherograms were assembled and edited in the Gap4 software of the Staden package (Staden 1996). The consensus sequences thus obtained had their identity confirmed using BLASTN (NCBI website) and were then aligned using the ClustalW algorithm, as implemented in Mega 6 software (Tamura et al. 2013). Each polymorphic site encountered along this alignment was individually checked and manually corrected, if necessary. For RHO, heterozygous sites were coded according to the nucleotide degeneracy/redundancy table and after unphased in DnaSP 5.10 software (Librado and Rozas 2009). This software was also employed to measure the minimum number of recombination events that best explains the diploid dataset.

The DnaSP 5.10 software was used to calculate the levels of genetic diversity within populations or population groups individually for each gene, as estimated from the average number of different haplotypes (H) or alleles (A) (Nei 1987), the average number of nucleotide differences between haplotypes or alleles (k) (Tajima 1983), the haplotype diversity (Hd) or expected heterozigosity (He) (Nei 1987), and the nucleotide (π) diversities (Nei 1987). The neutrality tests Tajima's (1989), and Fu's (1997) were performed in Arlequin 3.5 (Excoffier and Lischer 2010), with significance measured through 10,000 random permutations for both genes.

The relationships between haplotypes or alleles were inferred for both genes individually from networks generated by median-joining in the Network v.4.510 software (Bandelt et al. 1999). The levels of genetic differentiation among populations were measured for cyt *b* and RHO by the fixation index (FST) in Arlequin 3.5 using pairwise differences with 10,000 random permutations. A Mantel test was finally performed using these measures to check the correlation between the genetic (FST) and geographic distances.

For the concatenated dataset, different hypotheses of population groupings were assessed through spatial analysis of molecular variance (SAMOVA) (Dupanloup et al. 2002), in order to define the number and structure of the groups that are geographically homogeneous and maximally differentiated from each other. In this case, different hypotheses were evaluated through hierarchical analysis of molecular variance (AMOVA), as performed in Arlequin, with 10,000 permutations. The population structure and the most likely number of clusters of individuals were also assessed using the Bayesian Population Structure analysis performed in BAPS 6 (Corander et al. 2008).

The supermatrix phylogenetic tree was reconstructed using Bayesian analysis and maximum likelihood estimates in MrBayes 3.2.6 (Ronquist et al. 2012) and RaxMLHPC (Stamatakis 2014), respectively, under the partitioning scheme and substitution models suggested by PartitionFinder 1.1.1 (Lanfear et al. 2012). The Markov Chain Monte Carlo (MCMC) of the BA was run for 10,000,000 generations, sampling trees every 1000 generations, and burning 25% of the initial results. The ML search was performed under the new rapid hill-climbing algorithm, with 1000 bootstrap replicates. These analyses were performed without the use of outgroups, and the phylogenetic trees were further visualized and edited in FigTree 1.4.3 (Rambaut and Drummond 2009) as radial phylograms.

Additionally, a chronophylogenetic tree was reconstructed under a Bayesian approach (BA) in Beast 1.5.4 (Drummond and Rambaut 2007), using cyt *b* and RHO sequences with unlinked substitution and clock models. In this case, an uncorrelated lognormal relaxed molecular clock analysis was performed with a mean rate of 2% per million years (SD = 0.5%) for cyt *b*, as adjusted for ectotherms mitochondrial genomes (Brown et al. 1979). This prior was further complemented by the dating of 325 kyr (SD = 12.5 kyr) to the major split between oriental and occidental lineages, as recovered in the first Bayesian search conducted with MrBayes (see results). The analysis encompassed two independent runs of 20,000,000 generations, with trees and parameters sampled every 2000 iterations, and a burn-in of 25%. Results of each run were visualized in Tracer 1.7 (Rambaut et al. 2018) to ensure that stationarity was achieved and that convergence was reached. Posterior probabilities and the maximum credibility tree were inferred using TreeAnnotator 1.5.4 (Drummond and Rambaut 2007) and further visualized and edited in FigTree 1.4.3.

Finally, to estimate the effective number of females of each population group, a Bayesian analysis based on coalescence was implemented in LAMARC 2.1.10 (Kuhner 2006) under the GTR nucleotide substitution model, with effective population sizes set to 1 and 4, and relative mutation rates set 1 and 0.5 for the mitochondrial and nuclear partitions, respectively. Runs consisted of four simultaneous searches, each with 100 initial and four final chains, with a minimum of 1000 and 10,000 recorded parameter sets, respectively, and sampling every 20 generations after a burn-in of 1000 genealogies. Only population groups with more than 10 sampled individuals were used in this analysis.

Morphometry analysis

A set of 14 landmarks were defined and digitalized using TpsDig2 software ver. 2.26 (Rohlf 2016) (Supporting Information Fig. 1S). The coordinates were aligned using a Generalized Procrustes Analysis (GPA), where non-shape variability was removed after minimizing the differences in translation, scaling and rotation. Due to sexual dimorphism, the analyses were performed independently for males and females.

Shape variations among individuals of the different groups were first evaluated through an exploratory Principal Component Analysis (PCA), followed by MANOVA and pairwise MANOVA. Afterwards, a Canonical Variate Analysis (CVA) was employed to find the linear combination of predictors that best discriminates among groups. Cross validation test was used to measure the accuracy of correct classifications. These analyses were permormed in R (R Core Team 2017), using Geomorph (Adams and Otarola-Castillo 2013) and Vegan packages (Oksanen et al. 2017).

Results

In this study, sequences spanning 798 bp of the mitochondrial cyt *b* gene and 821 bp of the nuclear RHO gene were characterized, respectively, for 122 and 110 individuals of *A. wolterstorffi* collected at 22 different localities (Fig. 1) (Supporting Information Tables 1S and 2S). The intraspecific matrix of cyt *b* encompassed 39 different haplotypes presenting 65 variable sites, of which 48 were parsimoniously informative. For RHO, only four alleles were detected, and variability was restricted to three sites.

Molecular analyses

Cyt b dataset

Concerning cyt *b* diversity estimates, the number of haplotypes per sampling locality ranged from 1 (Sal, 33-INIA, 33-Pas, SJN-4, Buj, Pel-S, PN-RG and TAIM) to 6 (Tav and IL-RG), with minimum Hd and π of 0 (Sal, 33-INIA, 33-Pas, SJN-4, Buj, Pel-S, PN-RG and TAIM), and maximum Hd and π values of 0.889 (IL-RG) and 0.006 (SJN-3), respectively (Table 3S). None locality presented significant deviations from neutrality (Table 3S). A total of 111 cases of significant genetic structure were detected in the pairwise comparisons between populations, and these Fst values varied from 0.20 (in the comparison between IL-RG and Pel-P) to 1.00 (in the comparison between SJN-4 and Sal, 33-INIA, Buj and between Buj and 33-INIA) (Table 4S). The Mantel test indicated a significant correlation between genetic and geographic distances ($r = 0.52$; $p < 0.000$).

The haplotype network reconstructed with cyt *b* sequences revealed the presence of six independent haplogroups (Fig. 1a): (1) Eldorado, with haplotypes sampled at the municipality of Eldorado do Sul, RS, Brazil; (2) Pelotas, clustering haplotypes sampled in seven localities of Pelotas and Rio Grande, RS, Brazil; (3) Uruguay, grouping together the haplotypes sampled in the nine localities of Uruguay; (4) Tavares, with haplotypes sampled at Tavares, RS, Brazil; (5) São José do Norte (SJN), with haplotypes sampled at Cachoeirinha, RS, Brazil and in three localities of SJN, RS, Brazil; and (6) São José do Norte 2, with haplotypes sampled at one locality of SJN, where haplogroup 5 was also collected. No haplotype was shared between these haplogroups, and a star-like pattern of ramification could be evidenced for Uruguay (Fig. 1a).

RHO dataset

The pattern detected for RHO is less informative, since this marker revealed to be highly conserved in *A. wolterstorffi*. In this case, more than a single allele was detected only for SJN-3, SJN-4, Buj, Tav, Pel-P, PN-RG, IL-RG, IL2-RG and MP-RG, and the higher values of He and π were 0.644 (SJN-4) and 0.0009 (SJN-4), respectively (Table 5S). None locality presented significant deviations from neutrality (Table 5S), and significant Fst values were restricted to the comparisons

involving some Uruguayan and Brazilian localities (Table 6S), which resulted in a significant correlation between genetic and geographic distances in the Mantel Test ($r = 0.20$; $p < 0.018$). Even so, in the Network, none clear subdivision could be detected, and a starlike pattern was recovered for the species considered as a whole (Fig. 1b).

Concatenated dataset

Despite the differences in variability levels, as cyt *b* and RHO presented similar divergence patterns in the target species, they were jointly considered in the remaining analyses. The population structure suggested by SAMOVA for the supermatrix dataset lead to a plateau in FCT values when k was set to six, which subdivided *A. wolterstorffi* into the same population groups previously recovered in the cyt *b* Network: Pelotas, Uruguay, Tavares, São José do Norte, Eldorado and São José do Norte 2. In fact, the AMOVA performed with this structure revealed that the six groups of populations were able to explain 75.95% of the variation encountered in the concatenated dataset (Table 1). Bayesian clustering approaches implemented through the spatial BAPS model also supported the same structure.

Accordingly, the topology of the radial phylogram also recovered a similar structure, and all the population groups were recovered as reciprocally monophyletic in both BA and ML trees, with support values higher than 0.9 and 63, respectively (Fig. 2). These lineages grouped into two main clusters encompassing populations inhabiting drainages at the western (population groups of Pelotas, Uruguay, and Eldorado) and eastern (Tavares, São José do Norte and São José do Norte 2) margins of the Patos Lagoon. After setting this divergence to a time prior of 325 kyr (SD = 12.5 kyr) in a chronophylogenetic analysis, diversification of these clades was dated to approximately 222 and 186 kyr, respectively (Fig. 3). In this case, the Eldorado and Tavares population groups seem to have encompassed the most ancient divergences, respectively, although support for this position was tenuous, especially for the last. Diversifications of each of the six population groups were set between 104 and 7 kyr (Fig. 3).

Morphometric analyses

Applying the subdivision in six population groups, significant differences were found in the shape patterns for both, males and females ($p < 0.01$). Among females, most pairwise comparisons involving the population groups of Eldorado and Uruguay evidenced significant differentiation, although for males such a result was restricted to two comparisons involving Uruguayan specimens (Table 7S). In this sense, the PCA for males and females also suggested the existence of some differences between groups, especially in regard to the insertion of the caudal fin and to the position of the dorsal fin, respectively (Fig. 4a, b). For males, the PC1 explained 29.5% of shape variation, whereas PC2 explained 27.1%. For females, PC1 and PC2 explained 22 and 19.2% of shape variation, respectively. Nevertheless, when a CVA was used with the four population groups that had at least six individuals sampled, morphological differentiation between the different population groups became more evident (Fig. 4c, d). In this case, the overall classification accuracy reached 59.5 and 60.9% for males and females, respectively.

Diversity and differentiation evaluations in face of the new subdivision

As the subdivision among six population groups was consistently defined by the network of cyt *b*, and by SAMOVA, spatial BAPS, and the phylogenetic analyses of the concatenated dataset, this

Table 1 Analysis of molecular variance (AMOVA) performed with the concatenated dataset, following the subdivision proposed by spatial analysis of molecular variance (SAMOVA) with k set to six

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
Among groups	5	879.955	4.47445 Va	75.95
Among populations, within groups	16	77.258	0.45024 Vb	7.64
Within populations	222	214.558	0.96648 Vc	16.41
Total	243	1171.770	5.89117	

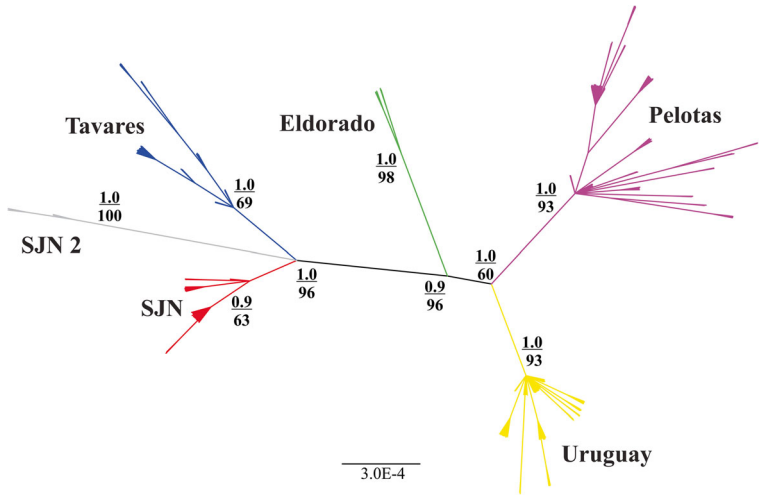

Fig. 2 Radial phylogram of the concatenated sequences, with internal node labels representing support values, given by posterior probabilities and bootstrap values in BA and ML evaluations, respectively. Branch lengths are proportional to the number of substitutions per site, and colours refer to population groups (see Fig. 1)

structure was further employed in new neutrality, diversity, and genetic differentiation tests. In general, at least two haplotypes were sampled for cyt *b* in each of the six population groups, which always presented haplotype diversity (Hd) values higher than 0.60 for this marker (Table 2). For rhodopsin, the population groups of Eldorado, SJN2 and Uruguay were fixed for a single allele, and the highest value of He was 0.54 for Tavares (Table 2). Conversely, the nucleotide diversity values (π) were generally moderate to low, ranging from 0.010 to 0.058 and 0.0004 to 0.0007 for cyt *b* and RHO, respectively. Regarding the neutrality tests, significant negative results were only obtained by cyt *b* for the populations of Pelotas and Uruguay (Table 2).

When testing genetic differentiation levels for cyt *b*, most comparisons between population groups presented significant genetic differences (Table 3, below the diagonal). The sole comparison that did not present a significant FST value was between Eldorado and SJN 2, which is probably an outcome of reduced sampling size. In all the significant comparisons, FST values between groups were higher than 0.7, indicating the presence of high levels of genetic differentiation (Table 3, below the diagonal). For RHO, significant genetic differences were only detected between the Uruguayan and Brazilian groups (Table 4, below the diagonal). The net Tamura 3-parameters distances also supported such a pattern, and the six lineages differed by a minimum of 0.9% (as seen between Tavares and SJN) and a maximum of 2.6% (as seen between groups Pelotas and SJN 2) for cyt *b* (Table 3, above the diagonal). Nevertheless, net distances between groups were zero when only RHO sequences were analysed (Table 4, above the diagonal).

Population parameters inferred for the concatenated dataset in LAMARC revealed theta values ~12.4-fold higher for Pelotas than for SJN, whereas the populations of Uruguay and Tavares presented similar intermediate theta values (Table 5). Even so, Bayesian analysis recovered effective numbers larger than 11,000 for all populations (Table 5).

Discussion

It is generally assumed that annual fish are subject to the concomitant action of a set of environmental, demographic, and metabolic conditions commonly associated with high evolutionary rates (Whitlock 2000; Loureiro 2004), which can lead to a great variability among populations. In agreement with this, our analyses have shown that, in addition to several cases of significantly high differentiation levels between individual populations, in an isolation-by-distance divergence pattern, *A. wolterstorffi* is subdivided into at least six different groups of populations. Such a structure was evidenced by the Network of cyt *b*, and by SAMOVA, BAPS, and the phylogenetic analyses of the concatenated dataset, and further supported by shape variation. So, despite the differences in evolutionary rates presented by the mitochondrial and nuclear markers, which can be attributed to distinct mutation rates, modes of inheritance and population sizes (Avise 2004; Templeton 2006), this subdivision was able to explain more than 75% of the variation detected for this species in both of the sampled

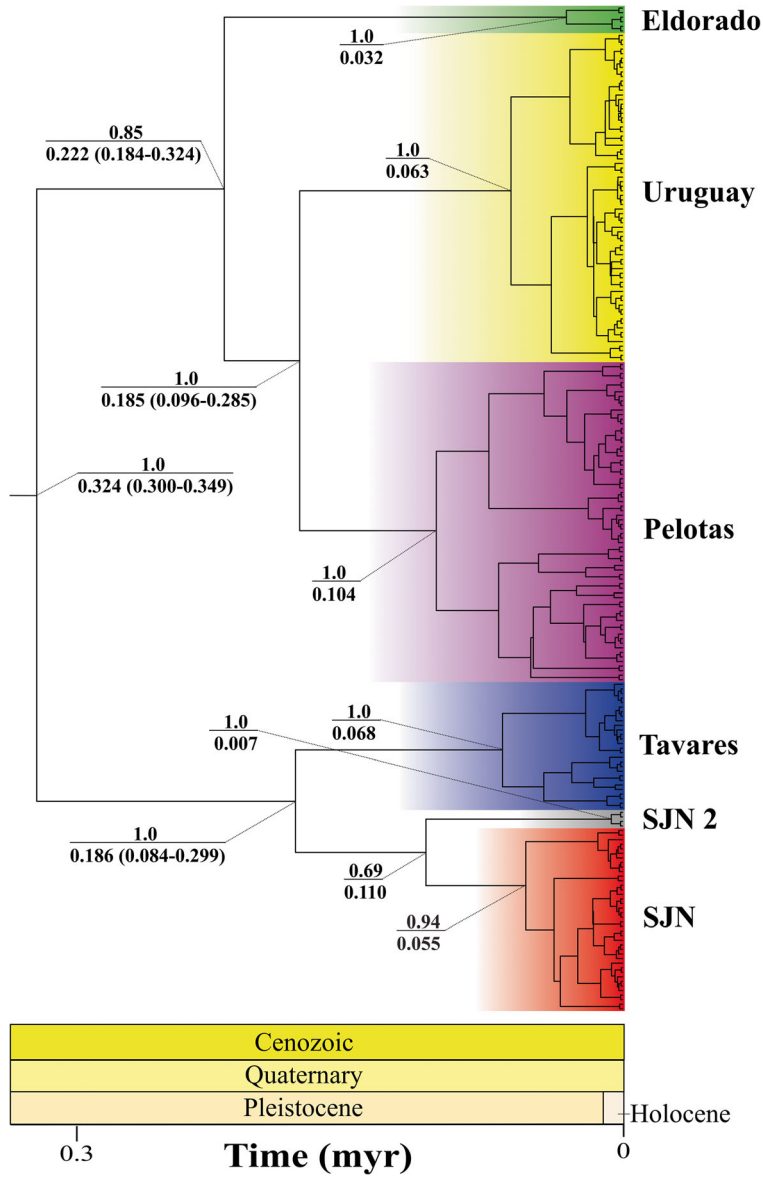

Fig. 3 Bayesian chronophylogenetic tree based on cyt *b* and RHO sequences sampled for *A. wolterstorffi*. Values above internal branches represent support values, given by posterior probabilities values in BA analysis. Values below nodes indicate divergence time estimates, with the highest posterior density (HPD) interval containing 95% of the sampled values presented within brackets for some of the detached clades. Groups of populations were represented by their respective names and colours (see Fig. 1). This tree was rooted with cyt *b* and RHO sequences of *A. nigrofasciatus*, *A. minuano*, and *A. adloffi*

loci. Moreover, cross validation tests performed with discriminant CVA resulted in overall classifications accuracies ranging 60%, evidencing an incipient morphometric differentiation between at least some of the evaluated groups. This suggests that vicariance has played an important role in the diversification of *A. wolterstorffi*, as also reported for several other Aplocheilidae species (Jowers et al. 2008; García et al. 2009, 2012, 2015; Bartáková et al. 2013; Ponce de León et al. 2014; Loureiro et al. 2015).

Although allopatric fragmentation seems to be an ongoing process within *A. wolterstorffi,* some level of gene flow seems to occur, especially at short distances, encompassing mainly populations located within each of the population groups. In fact, all FST values recovered in pairwise comparisons involving the six groups were significant and high, and they differed by a minimum of 0.9% corrected cyt *b* distances (with a maximum of 2.6%). These results, added by the shape divergence patterns and the evidence of recent diversification (see below), lead us to conclude that at least some of these groups may constitute incipient species. In fact, distances as small as 1.4% were previously reported for cyt *b* between different species of *Austrolebias* (García

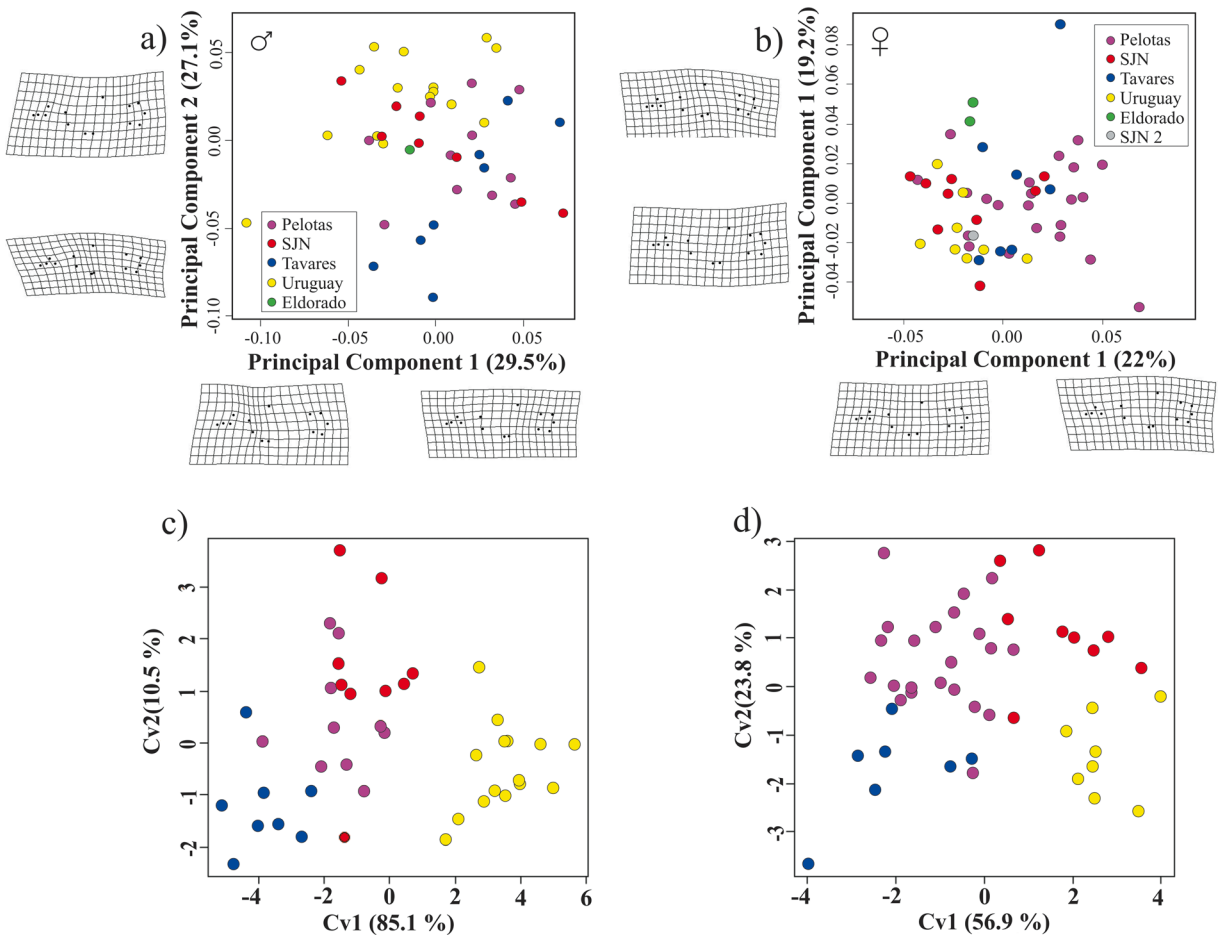

Fig. 4 Plots of Principal Component Analysis (PCA) (**a** and **b**) and Canonical Variate Analysis (**c** and **d**) showing the distribution of variation in *A. wolterstorffi* morphometric patterns: (**a** and **c**) males, (b and d) females. For the PCA plots, extreme PC values are represented by fishes' warped drawings on grid deformations. Colours refer to the different population groups (see Fig. 1)

Table 2 Genetic diversity estimates and neutrality tests of each of the six population clusters suggested in this study (see Results) for *A. wolterstorffi*

Groups	Cyt *b*						RHO					
	N	H	Hd	π	Neutrality tests		N	A	He	π	Neutrality tests	
					Tajima's D	Fu's F					Tajima's D	Fu's F
Pelotas	40	15	0.888 ± 0.0321	0.058 ± 0.033	**−1.5353**	−3.584	70	2	0.358 ± 0.055	0.0004 ± 0.0001	0.89601	1.417
Uruguay	39	9	0.789 ± 0.046	0.019 ± 0.014	−1.3892	**−3.526**	72	1	0	0	NC	NC
Tavares	17	6	0.713 ± 0.108	0.032 ± 0.023	0.4712	−0.128	28	3	0.542 ± 0.086	0.0007 ± 0.0001	0.38476	0.412
SJN	20	5	0.600 ± 0.101	0.016 ± 0.013	−0.1381	−0.933	40	4	0.477 ± 0.082	0.0006 ± 0.0001	−0.56317	−0.886
Eldorado	3	2	0.677 ± 0.314	0.020 ± 0.021	NC	1.061	6	1	0	0	NC	NC
SJN 2	3	2	0.667 ± 0.314	0.010 ± 0.013	NC	0.201	4	1	0	0	NC	NC
Total	122	39	0.951 ± 0.008	0.149 ± 0.076	−0.6296	−6.667	220	4	0.299 ± 0.037	0.00038 ± 0.00005	−0.6059	−1.126

N, the number of sequences; H, number of haplotypes; A, number of alleles; Hd, haplotype (gene) diversity; He, expected heterozygosity; π, nucleotide diversity (per site); Tajima's D and Fu's Fs, neutrality tests. The values in bold indicate significant measures ($P < 0.05$)

Table 3 Pairwise fixation indices (FST) (below the diagonal) and net Tamura 3-parameters distances (above the diagonal) of cyt *b* between the six population groups suggested in this study for *A. wolterstorffi*

Groups	Pelotas	Uruguay	Tavares	SJN	Eldorado	SJN 2
Pelotas	0	0.013	0.019	0.019	0.015	0.026
Uruguay	0.74075*	0	0.017	0.016	0.013	0.020
Tavares	0.77765*	0.87547*	0	0.009	0.018	0.017
SJN	0.81055*	0.90051*	0.75729*	0	0.017	0.014
Eldorado	0.70103*	0.86887*	0.83745*	0.91507*	0	0.023
SJN 2	0.82251*	0.92041*	0.83515*	0.90392*	0.9434	0

The asterisks indicate significant differences ($P < 0.05$)

et al. 2000), with even lower distance ranges (0 to 1.8%) being recently reported within the *A. bellottii* species complex (García et al. 2015). Considering that DNA-based approaches previously unravelled several unrecognized lineages of Neotropical annual fishes (Costa and Amorim 2011; Costa 2013; Costa et al. 2014, 2016, 2017), we argue here that *A. wolterstorffi* may in fact constitute a species complex. Nevertheless, it is likely that this complex presents a continuum of differentiation, in which at least some of the lineages might have yet reached a speciation endpoint, given by establishment of complete reproductive isolation.

Independent of the taxonomic status assigned to each of the six evolutionary units detected here, it is important that independent conservation strategies are applied to each these major sampling areas, four of which are located in Brazil. Although the levels of genetic diversity encountered for *A. wolterstorffi* as a whole (or for each of the individual population groups) were relatively high, this is likely explained by small-scale events of gene flow within population groups or even by the putative higher mutational rates previously assigned to mitochondrial genes in *Austrolebias* (García et al. 2015). In fact, similar levels of intrapopulation diversity were previously reported for several other species of annual fishes inhabiting different regions (García et al. 2000, 2015; Bartáková et al. 2013).

Nevertheless, the fact that each of these areas embraces an independent genetic stock calls attention to the need for rapid interventions, principally in the face of the rapid fragmentation and degradation of wetlands within the Brazilian territory (Volcan et al. 2015). This situation might decrease effective population sizes and gene flow, enhancing the action of random genetic drift and inbreeding and increasing the risk of mutational meltdown and inbreeding depression, respectively (Frankham et al. 2013). Likewise, the periodic flooding of rivers and lagoons within the distribution range of *A. wolterstorffi* might also threaten the persistence of each of these independent evolutionary units. In this sense, signs of population admixture were encountered for the population of São José do Norte, which presents two different haplogroups differing by a minimum of 11 mutational steps and assigned to different population groups. This result led us to hypothesize that the SJN2 individuals might represent immigrants from a distinct unknown population. Secondary colonisations might also be invoked to explain the presence of individuals

Table 4 Pairwise fixation indices (FST) (below the diagonal) and net Tamura 3-parameters distances (above the diagonal) for rhodopsin between the six population groups suggested in this study for *A. wolterstorffi*

Groups	Pelotas	Uruguay	Tavares	SJN	Eldorado	SJN 2
Pelotas		0	0	0	0	0
Uruguay	0.22010*		0	0	0	0
Tavares	0.05240	0.26234*		0	0	0
SJN	−0.00574	0.18247*	0.00455		0	0
Eldorado	0.06203	0	0.02251	0.00122		0
SJN 2	0.01984	0	−0.02711	−0.04696	0	

The asterisks indicate significant differences ($P < 0.05$)

Table 5 Bayesian posterior distribution of population genetic parameters inferred for the six population groups of *A. wolterstorffi* with cyt *b* and rhodopsin

	MPE	90% CI
Theta		
Pelotas	0.0087	0.0001–0.0108
Uruguay	0.0020	0.0001–0.0032
Tavares	0.0011	0.0006–0.0016
SJN	0.0007	0.0003–0.0012
Nef		
Pelotas	143,279	977–177,787
Uruguay	32,623	215–53,066
Tavares	18,311	9869–25,541
SJN	11,230	5344–20,197

MPE, most likely estimate; Nef, effective number of females; CI, confidence interval

from the SJN haplogroup in Cachoeirinha, a municipality located northwest of the Patos Lagoon. So, although current estimates of the effective population sizes do not suggest an incipient risk of extinction for any of the sampled population groups (see Frankham et al. 2004), and none of the populations presented significant signs of genetic bottlenecks, this could change rapidly and it is important that interventions are performed before the evolutionary potential of the populations is lost.

Finally, regarding the spatio-temporal evolutionary scenario, it is possible to infer that the six groups of populations encompass two distinct lineages, whose distribution coincides with the eastern and western margins of the Patos Lagoon, and suggest a north-south colonization route. Using the onset of the formation of the Patos Lagoon, related to the occurrence of the second Pleistocene Lagoon Barrier Depositional System and dated to approximately 325 kyr (Villwock and Tomazelli 2007) as prior to the divergence time between Oriental and Occidental lineages, together with the mean mitochondrial ectothermic rate of 2% per million years (Brown et al. 1979; Avise 1994), it was possible to infer that the first divergence within these two clades occurred around 222 (western) and 186 (eastern) kyr, compatible with range expansions enabled by the second Pleistocene Lagoon Barrier Depositional System related to the paleogeographical evolution of the South American Coastal Plain (Montaña and Bossi 1995; Tomazelli and Villwock 2005; Villwock and Tomazelli 2007). Interestingly, for the western clade, the lineage with the further north distribution encompasses the early offshoot, which is also in agreement with the paleogeographic history of the region (Villwock and Tomazelli 2007). Furthermore, the diversification of each of the six population groups seem to have occurred around 104 kyr (Pelotas population group) and 7 kyr (SJN2), and it is tempting to speculate that at least some of these events were related to the third or fourth Lagoon Barrier Depositional Systems, dated to approximate 120 kyr and 18 kyr to near the present, respectively (Villwock and Tomazelli 2007). The Uruguayan population group, in particular, inhabits a geologically older formation, which seems to have been recently colonized (about 63 kyr). In agreement with this, this lineage presents significant signs of population expansion, as given by the Fu's FS test and by the star-like network pattern recovered with the cyt *b* dataset, and by the lower diversity values revealed by RHO. More subtle signals of demographic or spatial expansions were also detected for the species complex taken as a whole (as given by the star-like pattern recovered with RHO), and for the Pelotas population group (as given the Tajima's D test performed with cyt *b*).

The well-registered paleogeoclimatic history of the South American Coastal Plain (Montaña and Bossi 1995; Tomazelli and Villwock 2000, 2005; Villwock and Tomazelli 2007) and the clear vicariance between Oriental and Occidental lineages of *A. wolterstorffi* also allowed us to use this divergence to calibrate our relaxed molecular clock analysis and to establish a new calibration system to the group. Until now, the most widely used method to date divergence events in the group employed the mean substitution rate for mitochondrial genes that was established by Brown et al. (1979) and adjusted by Avise (1994) for ectothermal species taken as a whole (García et al. 2012, 2014, 2015). As molecular clock substitution rates need to be calibrated for each gene in each studied lineage, the use of this molecular clock calibration system will allow the estimation of substitution rates for several other genes within the genus, enabling refinement of several questions related to the spatio-temporal evolutionary history of this and several other species of annual fish.

So, although this study clearly helped to enhance knowledge about the evolution of *A. wolterstorffi*, evidencing some hidden taxonomic and conservation problems, it should be followed by additional studies and initiatives aimed at the conservation of annual fish, which encompass one of the most threatened vertebrate

groups in Brazil (ICMBio 2013; Volcan et al. 2015). It is important that the knowledge generated here be used to help in the promotion and implementation of environmental education programs and to encourage the creation of protected areas. Only the adoption of a whole set of actions can help in the conservation of this fascinating and biologically unique group of species.

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