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

Conservação de *Austrolebias minuano*, peixe anual endêmico da planície costeira do Rio Grande do Sul ameaçado de extinção

Murilo de Oliveira Fernandes

Orientador: Prof. Dr. Antonio Sergio Varela Junior
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Aos meus pais, ao Felipe e aos verdadeiros
amigos que sempre torceram por mim.
Dedico esta conquista.

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RESUMO

A vulnerabilidade dos ambientes aquáticos sazonais, devido à ação antropogênica, tem levado à perda da diversidade e do patrimônio genético existente. Neste contexto, os peixes anuais representam 30% da ictiofauna ameaçada no Brasil e são totalmente dependentes da integridade física do seu biótopo. Dentre esses peixes, *Austrolebias minuano* é endêmica e considerada ameaçada de extinção devido à perda e degradação do seu habitat. Para estabelecer estratégias de conservação, estudos que visem compreender os níveis e a distribuição geográfica da variação genética são necessários. A partir deste conhecimento, pode-se, por exemplo, escolher as populações mais adequadas para a criopreservação espermática, uma estratégia que visa a estocagem e manutenção do material genético. Assim, o presente estudo tem como objetivo desenvolver a aplicação de potencial estratégia de conservação *in situ* e *ex situ* de *A. minuano*, a partir do conhecimento dos padrões de distribuição da diversidade genética da espécie. A fim de inferir potenciais populações-alvo para conservação, padrões de distribuição da variabilidade genética foram mensurados utilizando dois genes mitocondriais (cyt *b* e CO1) e um gene nuclear (ENC1) em seis populações de *A. minuano*. Também foram avaliados os efeitos de diferentes crioprotetores intracelulares em diferentes concentrações nos parâmetros de qualidade espermática. As análises genéticas mostraram que *A. minuano* pode ser dividida em pelo menos duas espécies, uma das quais contém a população do espécime tipo da espécie e pode ser considerada *A. minuano* sensu stricto. No processo de criopreservação, essa população foi escolhida e o crioprotetor metilglicol apresentou resultados promissores para ambos parâmetros avaliados. Assim, o uso da técnica de criopreservação se mostrou efetiva para a manutenção e armazenamento do material genético da espécie. *In situ*, no entanto, ficou claro que medidas individuais de preservação para todas as linhagens avaliadas são extremamente necessárias.

Palavras-chave: Biodiversidade; criopreservação; filogenia; killifish

ABSTRACT

The vulnerability of seasonal aquatic environments, due to anthropogenic action, has led to the loss of diversity and existing genetic patrimony. In this context, annual fish represent 30% of the ichthyofauna threatened in Brazil and are totally dependent on the physical integrity of its biotope. Among these fish, *Austrolebias minuano* is an endemic species and considered endangered due to the loss and degradation of its habitat. To establish conservation strategies, studies that aim to understand the levels and geographical distribution of genetic variation are necessary. From this knowledge, is possible, for example, choose the populations most suitable for sperm cryopreservation, a strategy that aims at the storage and maintenance of genetic material. Thus, the present study aims to develop the application of a potential in situ and ex situ conservation strategy of *A. minuano*, based on knowledge of the distribution patterns of the species' genetic diversity. In order to infer potential target populations for conservation, patterns of distribution of genetic variability were measured using two mitochondrial genes (cyt *b* and CO1) and one nuclear gene (ENC1) in six populations of *A. minuano*. The effects of different intracellular cryoprotectants at different concentrations on sperm quality parameters were also evaluated. Genetic analyzes showed that *A. minuano* can be divided into at least two species, one of which contains the population of the specimen type of the species and can be considered *A. minuano* sensu stricto. In the cryopreservation process, this population was chosen and the cryoprotectant methyl glycol presented promising results for both evaluated parameters. Thus, the use of the cryopreservation technique proved effective for the maintenance and storage of the genetic material of the species. In situ, however, it has become clear that individual preservation measures for all lineages evaluated are extremely necessary.

Key-words: Biodiversity; cryopreservation; killifish; phylogeny

APRESENTAÇÃO

Esta dissertação está dividida em três segmentos, o primeiro é referente à introdução geral sobre o tema proposto e às referências utilizadas. O segundo segmento é representado pelo primeiro capítulo, que caracteriza avaliações genéticas para escolha da população adequada para o procedimento de criopreservação, que será submetido na revista Reviews in Fish Biology and Fisheries, seguindo as normas da revista. O terceiro segmento apresentado é o segundo capítulo, abrangendo os procedimentos de criopreservação, onde o artigo será submetido à revista Aquaculture Research já formatado conforme as normas da revista.

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INTRODUÇÃO GERAL

1 Áreas úmidas

Devido às inúmeras tentativas de criação de uma definição para áreas úmidas, a Convenção de Ramsar de 1971 deliberou áreas úmidas como "extensões de brejos, pântanos e turfeiras, ou superfícies cobertas de água, sejam de regime natural ou artificial, permanentes ou temporárias, estancadas ou correntes, doces, salobras ou salgadas, incluindo as extensões de água marinha cuja profundidade na maré baixa não exceda os seis metros", sendo esta a definição mais aceita atualmente.

No estado do Rio Grande do Sul, as áreas úmidas vêm se destacando por sua elevada diversidade biológica (MITSCH; GOSSELINK, 2000). Esses ambientes são fonte de numerosos recursos naturais para a humanidade. Nesse sentido, esses ambientes vêm sendo intensivamente utilizados e modificados pela sociedade, em vista de seu uso na agricultura, nas indústrias, na recreação, no controle de inundações, no transporte, na purificação de dejetos (animais e industriais) e na geração de energia (BARON, 2002). A utilização exacerbada dos recursos hídricos e o impacto das mudanças climáticas vêm causando grandes alterações nesses ambientes (MALMQVIST; RUNDLE, 2002), tornando assim possível a extinção de muitos organismos (JUNK et al., 2014).

Dentre os tipos de áreas úmidas, os ambientes temporários, são considerados notadamente vulneráveis. No Rio Grande do Sul pequenas áreas úmidas temporárias são abundantes, especialmente na Planície Costeira (MALTCHIK et al., 2004) e abrigam inúmeras espécies ameaçadas de extinção. A perda de uma determinada espécie ocasiona não apenas um prejuízo ao patrimônio genético, mas uma interrupção na cadeia trófica, afetando gravemente a viabilidade dos diferentes ecossistemas. Compreendidos neste contexto, tem-se os peixes, que compõem grande parte da biodiversidade aquática e que, apesar de serem caracterizados como organismos com grande capacidade adaptativa, representam um dos grupos animais mais ameaçados de extinção (AGOSTINHO, 1999; DUDGEON et al., 2006; SILVA; DEUS; HILSDORF, 2006). Dentro deste grupo, se destacam os peixes anuais, que devido à perda e fragmentação de ambientes aquáticos temporários ou permanentes de água doce, vem sendo considerados em maior vulnerabilidade (FONTANA, 2003).

2 Peixes anuais

2.1 Caracterização, ciclo de vida, diversidade e distribuição

Peixes anuais incluem um diversificado grupo de peixes que chamam atenção pelo seu desenvolvimento e características evolutivas diferenciadas. Myers (1952), visando a inferência de um termo que abrangesse o ciclo de vida curto, deu o nome de "peixes anuais" aos vertebrados pertencentes a esse grupo. De acordo com Costa (2008), em peixes anuais, a ordem Cyprinodontiformes se encontra dividida em duas famílias taxonômicas, que ocorrem na África (Nothobranchiidae) e na América do Sul (Rivulidae). Evolutivamente, esses táxons são considerados como grupos irmãos, compartilhando muitas características fisiológicas, biológicas e ecológicas (COSTA, 1998). Espécies pertencentes a essas famílias exibem tamanho reduzido e geralmente são sexualmente dimórficas e dicromáticas, onde os machos costumam ser maiores, com nadadeiras mais desenvolvidas e padrões de cores mais atraentes (COSTA, 2008; REICHARD et al., 2009) (Fig. I).

Figura 1. Espécimes de *Austrolebias minuano* amostrados em charco localizado no município de São José do Norte, estado do Rio Grande do Sul. O macho da espécie (M) caracteriza-se pelos pares de linhas escuras em seu corpo e por ser maior em relação a fêmea (F).

São espécies que podem ser encontradas em ambientes aquáticos sazonais, brejos, charcos e lagoas rasas, que são formados somente em estações chuvosas e que permanecerão secos por um

período do ano (SÁNCHEZ et al., 2014). Uma das características mais importantes para a sobrevivência dos peixes anuais nesses ambientes é a capacidade de entrar em diapausa (MURPHY; COLLIER, 1997), característica observada nos seus ovos. Após atingirem a maturidade sexual, os adultos se reproduzem continuamente, e seus ovos são enterrados em até 15 cm de profundidade (VAZ-FERREIRA; SORIANO; SEÑORANS, 1966), ali permanecendo durante o período de seca, à espera da estação com as características adequadas para a eclosão e crescimento (Fig. II).

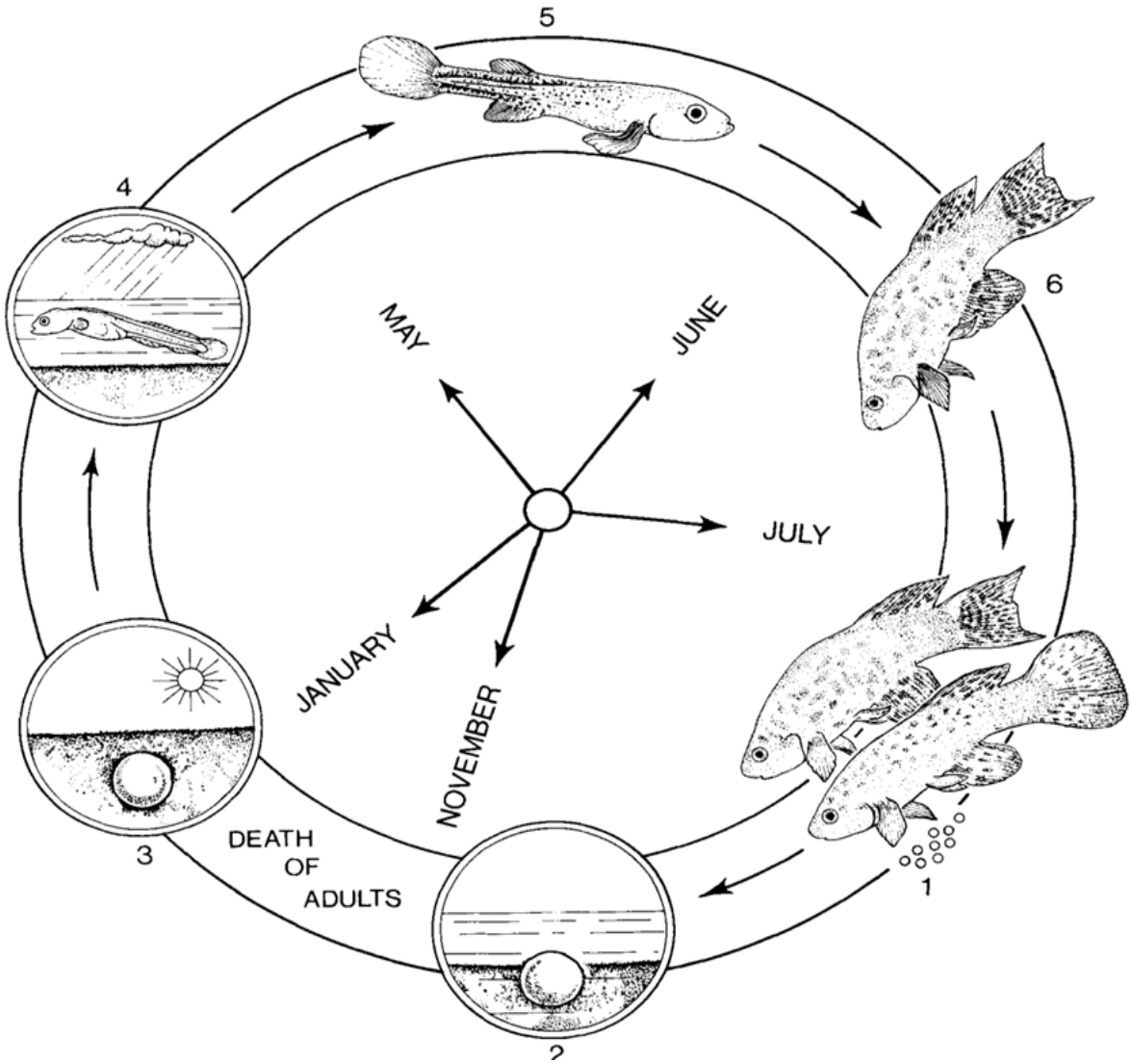

Figura 2. Esquema de ciclo de vida de *Austrolebias myersi* (Dahl, 1958), 1) entre os meses de julho a outubro, acontece o período de desova; 2) durante a desova, os peixes depositam seus ovos na porção de solo e estes permanecem em diapausa até o próximo período chuvoso; 3) no período de seca, todos os adultos morrem; 4) os ovos eclodem após a chegada de chuva no biótopo; 5) as larvas crescem de forma acelerada; 6) dentre a sexta e oitava semana os peixes anuais atingem a maturidade sexual. Fonte: Wourms (1972).

Costa (1998) ressalta que o aparecimento do ciclo de vida diferenciado (o anualismo) teve origem de forma independente nas famílias Nothobranchiidae e Rivulidae, sugerindo que essa homoplasia indicaria um processo de convergência adaptativa. Segundo o mesmo autor, o anualismo nos Rivulídeos está correlacionado com a colonização de biótopos periféricos em duas etapas, sendo primeiramente destacada sua ocorrência em riachos com pouca profundidade e poças permanentes, e logo em seguida em ambientes mais adjacentes e periféricos como charcos. Este mesmo autor argumenta que o anualismo constitui uma característica ancestral para Rivulidae, sendo o não anualismo verificado em determinados gêneros atuais visto como uma perda derivada do mecanismo de diapausa.

2.2 Família Rivulidae

Os rivulídeos pertencem à ordem Cyprinodontiformes, que conta com 10 famílias, 109 gêneros e 1013 espécies (BRAY, 2017). Rivulidae constitui a quarta maior família de peixes que ocorrem em ambientes de água doce da região neotropical, com cerca de 38 gêneros e 350 espécies descritas, sendo encontradas na América do Sul, Central e sul dos Estados Unidos (COSTA, 2008). Na América Central, se distribuem entre a região central do México e Panamá e ao longo das drenagens do Pacífico, na Costa Rica e no Panamá. Na América do Sul, a família é largamente distribuída, sendo encontrada do norte da Venezuela ao sul da Argentina, no Brasil, Paraguai e Uruguai. No entanto, a maior diversidade de rivulídeos é encontrada no Brasil, país que exibe cerca de um terço das espécies (COSTA, 2008). Volcan et al., (2015) destacam que pelo menos 37 espécies de Rivulidae são encontradas no estado do Rio Grande do Sul, Brasil, das quais 31 pertencem ao gênero *Austrolebias*.

Alguns autores afirmam que a combinação do ciclo de vida especializado, o tamanho corporal reduzido, a área de distribuição limitada, a baixa capacidade de dispersão e a ampla destruição das áreas úmidas, torna essas espécies mais vulneráveis à extinção (COSTA, 2008; COSTA et al., 2002; FONTANA; BENCKE; REIS, 2003; LANÉS, 2010; ROSA; LIMA, 2008). Conforme a IUCN (International Union for Conservation of Nature), no Livro Vermelho da Fauna Brasileira Ameaçada de Extinção, dentre as 135 espécies de peixes de água doce consideradas ameaçadas de extinção no Brasil, a maior parte (38,5%) é composta por indivíduos pertencentes à família Rivulidae (ROSA & LIMA, 2008). Além do mais, cerca de 70% das espécies de peixes de água doce ameaçadas de extinção no Rio Grande do Sul são rivulídeos (SEMA, 2014).

Considerando-se o *status* de ameaça apresentado por muitas espécies de rivulídeos, é fundamental que diferentes estratégias de conservação sejam adotadas, a fim de se criar possibilidades de preservar a diversidade de espécies. Visando definir parâmetros de conservação para as espécies brasileiras de peixes anuais, foi elaborado o PAN – Rivulídeos (Plano de Ação Nacional para a Conservação dos Peixes Rivulídeos Ameaçados de Extinção), que almeja estabelecer formas de proteção a esse grupo, tentando anular a perda de habitats (ICMBIO, 2012a), e tornando viável a elaboração de técnicas e estudos para garantir a preservação dos mesmos. Essas estratégias de preservação são, a grosso modo, distribuídas em conservação *in situ* e *ex situ*, de acordo com o sítio de manutenção do recurso preservado: enquanto que a primeira mantém o recurso genético protegido em seu local de origem, a segunda mantém o material genético preservado em um local fora da área de origem (CAROLSFELD et al., 2003). O PAN – Rivulídeos considera 52 espécies de peixes rivulídeos ameaçados de extinção no Brasil, sendo classificadas de acordo com os critérios da IUCN e publicadas no Volume II do Livro Vermelho da Fauna Brasileira Ameaçada de Extinção, em 2008. A lista contempla 64 espécies como focais na primeira edição do plano de ação (2012-2017), sendo 12 espécies pertencem ao gênero *Austrolebias*, a saber que *Austrolebias minuano* está contemplada para o segundo ciclo do plano (ICMBIO, 2012b).

2.3 Gênero Austrolebias

O gênero compreende indivíduos encontrados durante as temporadas de clima frio e alta precipitação na Argentina, Sul do Brasil, Paraguai, Uruguai e em alguns rios da Amazônia (LOUREIRO, 2015; NIELSEN e PILLET, 2015). Por serem espécies de periodicidade curta, os indivíduos de *Austrolebias* atingem a maturidade sexual em poucas semanas (ERREA; DANULAT, 2001; WALFORD; LIU, 1965), tipicamente em torno de 6 a 8 semanas (LIU; WALFORD, 1970). Segundo Costa (2006) e García (2006), *Austrolebias* apresenta cinco grupos de espécies, nomeados de acordo com a identidade de sua primeira espécie descrita (*A. robustus, A. elongatus, A. alexandri, A. bellottii e A. adloffi*), e três espécies basais (*A. luteoflammulatus, A. gymnoventris e A. jaegari*).

A maioria das espécies do gênero, tais como, *Austrolebias adloffi* Ahl, 1922 (COSTA & CHEFFE, 2001), *Austrolebias charrua* (COSTA & CHEFFE, 2001), *Austrolebias cyaneus* (AMATO, 1987), *Austrolebias jaegari* (COSTA et al., 2002), *Austrolebias litzi* (COSTA, 2006), *Austrolebias luteoflammulatus* (VAZ-FERREIRA; SIERRA; SCAGLIA, 1964), *Austrolebias melanoorus* (AMATO, 1986), *Austrolebias minuano* (COSTA & CHEFFE, 2001), *Austrolebias nachtigalli* (COSTA, 2006), *Austrolebias nigrofasciatus* (COSTA & CHEFFE, 2001),

Austrolebias paucisquama (FERRER; MALABARBA; COSTA, 2008), *Austrolebias prognathus* (AMATO, 1986), *Austrolebias univentripinnis* (COSTA & CHEFFE, 2005), *Austrolebias wolterstorffi* Ahl, 1924 (COSTA & CHEFFE, 2001), *Austrolebias pelotapes* e *Austrolebias pongondo* (COSTA et al. 2017), são encontradas, exclusivamente, no sistema hidrográfico Patos-Mirim.

Devido a diversidade de espécies, suas especificidades em relação ao seu habitat e à utilização de invertebrados como principal recurso alimentar, espécies de *Austrolebias* são de fundamental importância para o equilíbrio do ecossistema (VOLCAN et al., 2011). Segundo Furness (2016), por serem extremamente adaptadas e dependentes da integridade física do seu biótopo, essas espécies se tornam modelos evolutivos de estudo bioecológicos, relacionados a sua plasticidade fenotípica, tolerância fisiológica e alta capacidade adaptativa.

2.4 Austrolebias minuano

O grupo *A. adloffi* inclui oito especies de ocorrência restrita ao sul do Brasil (COSTA, 2006; VOLCAN, 2009), dentre as quais, *Austrolebias minuano*, que é considerada uma espécie de pequeno porte, cujos indivíduos tem, em média, 4,7 cm ao atingirem a maturidade sexual (6-8 semanas) (COSTA & CHEFFE, 2001). Na espécie é possível diferenciar o macho da fêmea através de características físicas bem delimitadas que evidenciam seu dimorfismo sexual. Enquanto que o macho apresenta em seu corpo pares verticais de listras, que se alternam entre um tom mais claro e um mais escuro de escamas, não se observa na fêmea a incidência do mesmo padrão de cores (Figura 1). De acordo com Costa (2006), esta espécie é endêmica de charcos do Sistema Lagunar Patos-Mirim, Rio Grande do Sul, tendo sido coletada previamente em charcos localizados nos municípios de Rio Grande, São José do Norte e Tavares. Assim como diversas outras espécies de *Austrolebias, A. minuano* figura nas listas nacionais e estaduais de espécies ameaçadas de extinção (FONTANA, 2003; ICMBIO, 2012b; SEMA, 2014), e é uma das espécies focais do PAN – Rivulídeos (ICMBio, 2012).

Recentemente, uma das populações previamente atribuídas à *A. minuano* foi descrita como uma nova espécie do grupo, *A. pongondo* (COSTA; CHEFFE; AMORIM, 2017). Esta parece exibir uma distribuição endêmica ao município de Rio Grande, e é morfologicamente distinta de *A. minuano* por apresentar múltiplas fileiras de pontos azuis e nenhuma zona cinzenta escura na porção posterior da barbatana dorsal (COSTA; CHEFFE; AMORIM, 2017).

3 Conservação

Ambientes temporários têm sido drasticamente destruídos, tanto para o uso agrícola como em áreas em processo de urbanização e desmatamentos. Devido às altas taxas de perda de componentes da biodiversidade e visando o incremento de esforços voltados à conservação dos recursos biológicos, são necessários estudos que visem mitigar os danos promovidos pela ação antrópica no ambiente (VOLCAN, 2009). Nesse sentido, a conservação *in situ* refere-se à conservação do habitat natural e a reconstituição de populações de espécies viáveis, permitindo a continuação dos processos evolutivos, enquanto a conservação *ex situ* envolve a manutenção fora do habitat e como principal característica a preservação genética por um período maior. Estes procedimentos são complementares e formam, estrategicamente, a base para a implementação da conservação da biodiversidade e uso dos recursos genéticos (BRASIL; MINISTÉRIO DO MEIO AMBIENTE - MMA, 2018).

3.1 Diversidade Genética

Uma linha de fatores, como taxas mutacionais, tamanho efetivo populacional e nível de fluxo gênico, está relacionada ao nível de diversidade genética apresentado por qualquer população ou espécie, bem como sua estruturação (FRANKHAM et al., 2004). Em conjunto, esses fatores podem reduzir a diversidade genética intrapopulacional, aumentando o risco de colapso mutacional (isto é, o declínio da taxa reprodutiva e espiral descendente em direção à extinção devido à fixação casual de novas mutações ligeiramente deletérias em populações pequenas) e de depressão por endocruzamento (redução na reprodução, sobrevivência ou outros caracteres quantitativos devido à endogamia) (FRANKHAM et al., 2004). Segundo o mesmo autor, todos esses padrões e processos são extremamente importantes do ponto de vista da conservação, já que podem afetar drasticamente o potencial evolutivo de uma espécie. Portanto, o estabelecimento de qualquer estratégia de conservação deve ser precedido por estudos desenhados no sentido de compreender os níveis e a distribuição geográfica da variação genética dentro do grupo-alvo.

3.2 Criopreservação

Pesquisas relacionadas à criopreservação de gametas têm avançado exponencialmente ao longo dos últimos anos (CHAKRABORTY et al., 2011; FERREIRA; MONTENEGRO; SALMITO-VANDERLEY, 2018; HAGEDORN; CARTER, 2011; HOROKHOVATSKYI et al., 2018; MACHADO et al., 2018). Esse instrumento metodológico busca viabilizar a manutenção da

estrutura e da funcionalidade dos gametas, através do congelamento do material genético e do seu armazenamento em bancos de germoplasma (DAY; WALKER, 2007). A célula espermática é ideal para este tipo de pesquisa, por demonstrar resistência à refrigeração e congelamento (LABBE et al., 1997).

No âmbito da conservação, o uso da criopreservação permite a proteção dos recursos genéticos e conservação da biologia (FIGUEROA; VALDEBENITO; FARIAS, 2016), tornando possível evitar a perda de qualquer espécie com risco de extinção. Afinal, a disponibilidade de recursos genéticos criopreservados, como espermatozoides e oócitos, pode permitir a recuperação de espécies ameaçadas, ao evitar a perda de diversidade genética, e garantir a reintrodução de uma espécie no ambiente natural, possibilitando recuperar e preservar espécies ameaçadas de extinção (CABRITA et al., 2010; MARTÍNEZ-PÁRAMO et al., 2009).

Em peixes, antes de ser congelado, o sêmen necessita de uma diluição em soluções com crioprotetores e diluidores. Os crioprotetores são utilizados na técnica com o intuito de preservar as estruturas celulares e prevenir as crioinjúrias dos espermatozoides, podendo ser classificados como intracelulares (internos) ou extracelulares (externos). Exercendo o papel de penetrar no interior da célula, os crioprotetores internos interferem na formação de cristais de gelo que lesionam a membrana celular (FAUSTINO et al., 2010; VARELA JUNIOR et al., 2009; VIEIRA et al., 2011), impedindo a desnaturação do DNA (KODERLE et al., 2009) e lesões mitocondriais e nos microtúbulos (HAMMERSTEDT et al., 1992).

De acordo com Viveiros e Godinho (2008), os crioprotetores intracelulares mais utilizados para a criopreservação de gametas de peixes são dimetilsulfóxido (DMSO) (MERYMAN, 1971; THIRUMALA et al., 2006; VARELA JUNIOR et al., 2012; VIVEIROS; GODINHO, 2009), glicerol (VARELA JUNIOR et al., 2012), e metilglicol (MARIA et al., 2006). O DMSO tem evidenciado resultados significativos no congelamento de espermatozoides de peixes (MIRZOYAN et al., 2006; SUQUET et al., 2000; VARELA JUNIOR et al., 2012; ZILLI et al., 2014), pois penetra facilmente na membrana plasmática da célula espermática, por ser altamente solúvel em meio aquoso (MACGREGOR, 1967). Este crioprotetor possui a capacidade de interagir ou combinar com ácidos nucleicos, carboidratos, lipídeos, proteínas e muitas outras substâncias sem alterar de forma irreversível a configuração molecular (SOJKA; HOPE; PEARSON, 1990).

O Glicerol também é largamente empregado na preservação de gametas masculinos (ALVARENGA et al., 2005) e utilizado em criopreservação de algumas espécies de peixes, como o Tambaqui (*Colossoma macropomum*) (VARELA JUNIOR et al., 2012). Porém, este álcool tem evidenciado desvantagens quando comparado a outros crioprotetores, especialmente sobre a integridade das estruturas celulares, devido ao seu peso molecular alto, alta viscosidade e nível

elevado de impenetrabilidade (HAMMERSTEDT; GRAHAM; CAROLINA, 1992), possivelmente devido a sua toxicidade em altas concentrações e baixa mobilidade através das membranas (MERYMAN, 1971).

O metilglicol também tem mostrado eficiência como crioprotetor (MARIA et al., 2006; NASCIMENTO et al., 2010; VIVEIROS; GODINHO, 2009), apresentando sucesso no congelamento de sêmen de pirapitinga *Brycon nattereri* (MARIA et al., 2006) e Suruvi, *Steindachneridion scriptum* (PEREIRA et al., 2018). O metilglicol é derivado do metanol (CH3OH) e do óxido de eteno (CH2OCH2), tendo se mostrado menos tóxico para as células espermáticas de peixes submetidas a criopreservação (VIVEIROS; GODINHO, 2009).

Qualquer um destes crioprotetores, por sua vez, precisam ser diluídos para serem acrescentados ao sêmen a ser preservado (HOLT, 2000; VIVEIROS; GODINHO, 2009). Segundo Purdy (2006), o diluente tem por finalidade fornecer energia, proteger de danos relacionados com a temperatura e sustentar um ambiente adequado para os espermatozoides resistirem provisoriamente. O diluidor BTS® (Beltsville Thawing Solution) é comumente utilizado no resfriamento de sêmen suíno (VIVEIROS; GODINHO, 2009), e tem obtido sucesso também na preservação de sêmen de peixes (MURGAS et al., 2007; PEREIRA et al., 2018).

Já que a composição bioquímica do esperma varia amplamente entre espécies, devido às variações na quantidade de lipídios e açúcares responsáveis pelo metabolismo das células espermáticas, para cada espécie é necessário o desenvolvimento de um protocolo de criopreservação diferenciado (CAROLSFELD et al., 2003; SHIN, et al., 1988). Portanto, a toxidade dos diversos diluentes e crioprotetores são diferentes para cada espécie. Para se estabelecer um protocolo eficiente e prático para a criopreservação espermática, devem ser padronizados fatores principais como: tipo e concentração de crioprotetor, condições para congelamento e descongelamento do esperma, diluidores e tempo de cada processo. Neste processo de otimização, deve-se fazer a avalição de motilidade do espermatozoide, visando a observação da qualidade do mesmo a cada etapa, antes do congelamento e pós-descongelamento (ALAVI; COSSON, 2005).

Neste sentido, Ávila-Portillo (2006) afirma que durante a criopreservação espermática, a integridade da membrana plasmática é o parâmetro mais afetado, uma vez que mudanças na temperatura deixam as membranas em altos níveis de estresse. Segundo Aurich (2005), a deformação na membrana celular pode levar a perda da motilidade durante o processo de congelamento e descongelamento, o que constitui um dos mais preocupantes danos causados pelo processo de criopreservação espermática. De fato, pequenas mudanças na estrutura do esperma podem ocasionar a redução da motilidade, capacidade de deslocamento e até de fecundação do

ovo (LEBOEUF; RESTALL; SALAMON, 2000). Vale ressaltar que para que se obtenha sucesso com a técnica de criopreservação espermática, uma alta viabilidade espermática funcional é fundamental (ANDRABI, 2009).

Assim, apesar da sua grande valorização no mercado de produção animal (CASTAÑO VILLADIEGO et al., 2017) e da sua incipiente utilização no âmbito da conservação (SEKI; YOSHIZAKI, 2018; SILVA; KUHNEN; SANCHES, 2018), o armazenamento de sêmen pelo processo de criopreservação pode apresentar problemas em alguns reprodutores, cujos espermatozoides sofrem determinadas crioinjúrias ao longo das etapas do processo (TASSERON; AMIR; SCHINDLER, 1977). Essas crioinjúrias são ocasionas por interação entre as mudanças biofísicas, bioquímicas e ambientais ocorridas durante o processo de criopreservação espermática (FICKEL; WAGENER; LUDWIG, 2007), ou seja, essas células são expostas a estresses resultantes das mudanças de volume e consequentes alterações nas concentrações de íons e eletrólitos nas soluções intracelulares (STORNELLI; TITTARELLI; SAVIGNONE, 2005).

De acordo com Pesch e Bergmann (2006), as crioinjúrias podem ser classificadas de acordo com a ação direta dos cristais de gelo formados a partir do choque frio e da interação água/soluto na medida em que o gelo é produzido no meio. Neste mesmo sentido, Holt (2000) diz que uma taxa de refrigeração ótima deve ser lenta o bastante para prevenir a formação letal de cristais de gelo intracelular e rápida o suficiente para minimizar as injurias do tempo de exposição a altas concentrações de sais, devido ao comprometimento da sobrevivência (CURRY et al., 1996) e fertilidade (ARRUDA et al., 2007). Por outro lado, como durante o processo de descongelamento ocorre a inversão das mudanças ocasionadas pelos processos de resfriamento e congelação, com o decréscimo na concentração intracelular de solutos e a restauração da concentração de água e do volume celular (HOLTZ, 1992), torna-se imprescindível que a técnica de descongelamento seja determinada a partir da técnica de congelamento, evitando uma grande discrepância entre os processos (PURDY, 2006). Segundo Ávila-Portillo (2006), deve-se ainda dedicar uma especial atenção ao tempo de cada processo para se evitar a recristalização do meio interno.

Todos os parâmetros e variáveis de um protocolo de criopreservação (tipo de substância, concentração, tempo de ação, etc.) devem, portanto, ser cuidadosamente avaliados durante a criação ou otimização de um protocolo de criopreservação. Além disso, para fins de implementação da técnica, avaliações dos padrões de distribuição da diversidade genética dentro da espécie alvo são importantes, tanto para a escolha da(s) população(ões) a ser(em) criopreservadas, quanto na escolha das populações potencialmente beneficiadas pela mesma.

OBJETIVOS

OBJETIVO GERAL

- Realizar estudos técnicos que visem a definição e a proposição de estratégias de conservação para a espécie de peixe anual ameaçada de extinção *Austrolebias minuano*.

OBJETIVOS ESPECÍFICOS

- Avaliar os padrões de distribuição da diversidade genética da espécie, de modo a fornecer subsídios para a melhor aplicação do protocolo desenvolvido em futuros estudos;
- Elaborar um protocolo de criopreservação espermática para *A. minuano*.

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CAPÍTULO 1

Artigo a ser submetido na revista “Reviews in Fish Biology and Fisheries”

692 Murilo de Oliveira Fernandes (https://orcid.org/0000-0003-2030-4119), Daiana Kaster Garcez
693 (https://orcid.org/0000-0003-4526-9647), Antonio Sergio Varela Junior (https://orcid.org/0000-0003-
694 4901-5118), Lizandra Jaqueline Robe (https://orcid.org/0000-0001-8506-9143)

695

696 **Phylogeography of the endangered neotropical annual fish, *Austrolebias minuano***
697 **(Cyprinodontiformes: Rivulidae)**

698 M. O. Fernandes
699 D. K. Garcez
700 A. S. Varela Junior
701 Programa de Pós-Graduação em Biologia de Ambientes Aquáticos Continentais
702 (PPGBAC), Universidade Federal do Rio Grande (FURG), Rio Grande 96203-000, Rio Grande do
703 Sul, Brazil.

704

705 L. J. Robe*
706 Departamento de Ecologia e Evolução (DEE), Centro de Ciências Naturais e Exatas (CCNE),
707 Universidade Federal de Santa Maria – UFSM, Santa Maria, Brazil.

708

709 * Corresponding author
710 Email: lizbiogen@gmail.com (LJR)
711 Phone: +55 (53) 98419-5271

712

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Abstract

A portion of the ichthyofauna threatened in Brazil is composed of species that live in seasonal aquatic environments. Among them, the annual fish *Austrolebias minuano* lives in small ponds, encompassing unique and isolated populations that are endangered. Therefore, the objective of this study is estimating the levels of diversity and genetic structure within and between populations of *A. minuano*, seeking the reconstruction of its evolutionary history and establishing conservation strategies. Characterization of mitochondrial cyt *b*, CO1 and nuclear ENC1 genes sequences showed division of four lineages, which may constitute at least three different species: the already described *A. pongondo*, *A. minuano* sensu stricto and one new species that embraces two population groups inhabiting the eastern margin of the Patos Lagoon. The genetic distances between *A. pongondo* and *A. minuano* were as high as the distances between the eastern and western lineages of *A. minuano* sensu lato. All comparisons detected a significant genetic structure between these groups, which high levels of genetic differentiation and revealed reciprocally monophyletic. In the chronophylogenetic reconstructions, the species of western margin of the lagoon constituted the first to branch out while whereas the two lineages of the eastern margin diverged more recently. The divergence timings revealed that divergences occurred before the occurrence of the first Pleistocene Lagoon Barrier Depositional System related to the paleogeographic evolution of the South American Coastal Plain. Further studies, including morphological and genetic studies that included other species of the *A. adloffi* species group are imperative in order to refine this scenario.

Keywords

Annualism – Biodiversity – Killifish – Molecular phylogeny

Introduction

Among neotropical annual killifish, *Austrolebias* comprises 50 valid species (Alonso et al. 2018; Serra and Loureiro 2018) that inhabit seasonal ponds at the Southern portion of the Neotropics. Most of these species occur in association with two river basins, the Paraná-La Plata basin and the Patos-Mirim drainage system, with only one species registered on the Amazon basin (Costa 2006; Alonso et al. 2018). Since these species are extremely adapted and dependent on the physical integrity of their biotope, they become important evolutionary models of bioecological studies, related to phenotypic plasticity, physiological tolerance and adaptive capacity (Furness 2016). Moreover, populations of annual fish are usually small, isolated and restricted, these species become ideal models for studies in genetics and evolutionary biology (Berois et al. 2015).

The highly specialized life cycle of these fish, which present an extreme dependence on seasonal pounds and annual and non-overlapping generations times impose serious flaws to gene flow among populations, leading to an increase on the levels of inbreeding and genetic structuring. These mechanisms also tend to reduce genetic diversity, which may decrease the species' reproductive capacity and limit its ability to adapt (Beheregaray et al. 2016). At the same time, these mechanisms increase the divergence between populations, which may lead to morphological dissimilarity and result in events of allopatric speciation (de Sá et al. 2015). All these properties make this an extremely specious and diversified evolutionary lineage (Sedláček et al. 2014). Nevertheless, these same properties in combination with the limited distribution and the straight forward destruction of humid areas make these species especially vulnerable to extinction (Fontana 2003; Rosa and Lima 2008).

Austrolebias minuano (Costa and Cheffe 2001) is endemic to the Coastal Plain of Rio Grande do Sul, occurring in temporary wetlands of the Patos-Mirim Lagoon System. This species is included on the *A. adloffi* species group, which encompasses 11 species (Volcan et al. 2014; Costa et al. 2017) that are remarkable for their great sexual dimorphism (Costa 2006). However,

767 *A. minuano* differs from other species of the group, and even from most species of *Austrolebias*,
768 except *Austrolebias wolterstorffi,* since it is found on both margins of the Patos Lagoon (Figure
769 1A). The fact that this Lagoon usually encompasses a barrier to the dispersion of other killifish
770 (Garcez et al. 2018) and the endangered (EN) status occupied by *A. minuano* in local red lists of
771 threatened species (SEMA 2014) highlights the importance of addressing the potential of cryptic
772 diversity within this lineage. In fact, recently, a local population of *A. minuano* occurring on the
773 west margin of the Patos Lagoon was described as a new species (Figure 1A), named *A. pongondo*,
774 through molecular and morphological evidence (Costa et al. 2017).
775 The use of molecular procedures for studying the levels of diversity and structure between
776 populations of annual fish has already revealed an effective strategy in obtaining information for
777 the conservation of such species (Garcez et al. 2018). By estimating the population structure, for
778 example, a better targeting of conservation efforts can be made, aiming at a more effective use of
779 available resources (Beheregaray et al. 2016). In addition, assessing the levels of genetic diversity
780 presented by different populations can also provide significant estimates about the degree of
781 disturbance of their biotope (Matioli and Fernandes 2012). So, the aim of this study is to evaluate
782 the levels of genetic diversity and structure within and among populations of *A. minuano*, seeking
783 to assist in the reconstruction of its evolutionary history and in the establishment of management
784 and conservation strategies for the target species.
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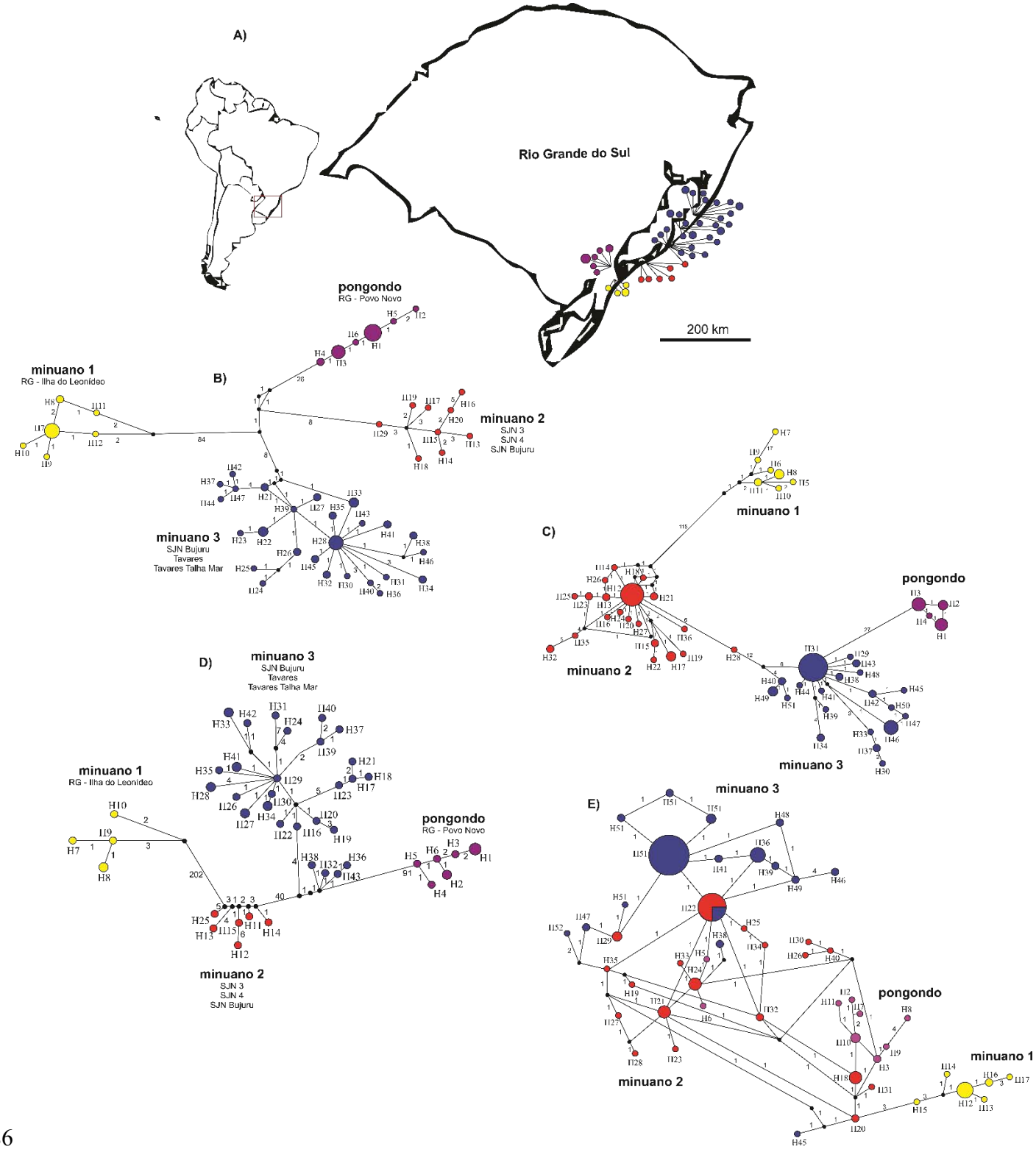


786

787 **Figure 1.** (a) Collection points and areas of occurrence recorded for *A. minuano* sensu lato (yellow) and *A. pongondo*
788 (purple). (b) Median-joining networks of the 47 cyt b haplotypes, (c) 51 CO1 haplotypes, (d) 43 CO1 and Cyt b
789 concatened haplotypes, and (e) 52 ENC1 alleles. The size of each circle is proportional to haplotype frequencies, and
790 colors refer to the main population groups recovered in this study (see Results).

Material and methods

Ethics statement

Methods used in this study were approved by the Ethics Committee for Animal Use of Federal University of Rio Grande (CEUA-FURG, permission number: 23116.008163/2015-23). Euthanasia methods followed the Federal Council of Veterinary Medicine (Resolution nº 05/2012 1000).

Study area

Individuals of *A. minuano* and *A. pongondo* were collected between 2015 and 2017 in six and one sampling locations, respectively, distributed in the Patos-Mirim lagoon system, in the southernmost Brazilian state of Rio Grande do Sul. This area encompasses all the sampling locations known for both species. All the sampled sites are within Quaternary sedimentary deposits of the coastal plain. This region was reworked during the paleoclimatic alterations 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).

Samplings

For this study, a total of 140 fishes were collected in temporary ponds, with the help of hand nets, after the reproductive season of 2015, 2016 and 2017, at the months of August and September. Fishes were euthanized with an overdose of 3,000 mg/L of eugenol anesthetic and fixed just after this procedure in 95% ethanol.

DNA sequences

Approximately 30 mg of muscular tissue dissected from the caudal peduncle was used to extract the total DNA, using a pre-established protocol (Sambrook J 1989). The obtained DNA was used in the amplification of approximately 770 bp of the mitochondrial cytochrome *b* (cyt *b*) gene, 1140 bp of the mitochondrial cytochrome c oxidase subunit 1 (CO1) and 620 bp of the nuclear ectoderm-neural cortex protein 1 (ENC1), using the primers L14735 (5' AAAAACCACCGTTGTTATTCAACTA 3') and CB3-H (5' GGCAAATAGGAARTATCATTC 3') (Wolf 1999; Palumbi et al. 2002), LCO1490 (5' GGTCAACAAATCATAAAGATATTGG 3') and COX1R (5' GG YTCTTCRAARGTGTGATAS 3') (Costa and Amorim 2011), and F85 (5' GACATGCTGGAGTTTCAGGA 3') and R982 (5' ACTTGTTRGCMACTGGGTCAAA 3') (Li et al. 2007), respectively.

Polymerase chain reactions (PCR) were performed 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. For the mitochondrial gene cyt *b*, thermocycling profile consisted of a first denaturing step of 5 min at 94 °C; 35 cycles of 45 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C; and a final extension step of 10 min at 72 °C. For the mitochondrial gene CO1 and the nuclear gene ENC1, the cycling conditions encompassed 50 s at 94 °C, 50 s at 54 °C and 1 min at 72 °C; and 30 s at 94 °C, 30 s at 53 °C and 30 s at 72 °C, respectively.

After confirming the success of the amplification, amplicons were purified with a solution of 7.5 M ammonium acetate ($C_2H_7NO_2$) and sent to be sequenced. Sequencing was performed in a Perkin-Elmer ABI Prism 377 Automated Sequencer (MACROGEN, Seoul, Korea) using the primers described above.

Data analysis

Software Gap-4 (Staden 1996) was used to edit and assemble the electropherograms. The consensus sequences thus obtained were aligned using ClustalW, as implemented in Mega 6

840 software (Tamura et al. 2013). Each individual polymorphic site encountered along this alignment
841 was checked and manually altered, if required. The heterozygous positions of ENC1 were further
842 unphased using DNAsp 5.10 (Librado and Rozas 2009).

843 First of all, relationships between haplotypes or alleles were inferred individually for each
844 gene through networks generated by median-joining in Network v4.510 (Bandelt et al. 1999).
845 Phylogenetic reconstructions were further performed for each gene through Bayesian Analysis
846 (BA), as performed by MrBayes 3.2.6 (Ronquist et al. 2012). This analysis was performed through
847 two runs of 10,000,000 generations of MCMC, sampling every 1000, according to the model
848 selected by an AIC test (Akaike 1974) implemented in jModelTest (Darriba et al. 2012).

849 Levels of genetic diversity within populations or population clusters were then estimated
850 using DnaSP 5.10, through the following parameters: average number of different haplotypes (H)
851 or alleles (A), average number of nucleotide differences between haplotypes or alleles (k),
852 haplotype diversity (Hd) or expected heterozygosity (He), and nucleotide (π) diversities. The
853 neutrality tests Tajima's D (Tajima 1989) and Fu's FS (Fu 1997) were performed in the same
854 software for each of the three genes, individually and in combination.

855 The level of genetic differentiation among populations or population clusters were
856 measured by the fixation index (FST) in Arlequin 3.5 using pairwise differences, with 10,000
857 random permutations. A Mantel test was performed using these measures to check the correlation
858 between genetic (FST) and geographic distances. Different hypothesis of population grouping
859 were further addressed through spatial analysis of molecular variance (SAMOVA) (Dupanloup et
860 al. 2002), which relies on hierarchical analysis of molecular variance (AMOVA), as performed in
861 Arlequin, and through a Bayesian Population Structure analysis, as performed in BAPS 6
862 (Corander et al. 2008).

863 Finally, a chronophylogenetic tree was reconstructed under a BA in Beast 1.5.4
864 (Drummond and Rambaut 2007), using unlinked nucleotide substitution models, unlinked

uncorrelated lognormal relaxed molecular clock models and a linked coalescent constant population size tree. In this case, a normal prior distribution with mean of 0.0273 and stdev of 0.00332 was used for the ucld.mean of cyt *b*, as recently adjusted for *A. wolterstorffi* by Garcez et al. (2018). The analysis encompassed 50,000,000 generations, with trees and parameters sampled every 1,000 iterations, and a burn-in of 10%. Results were visualized in Tracer 1.7 (Rambaut et al. 2018) to evaluate convergence and stationarity and to ensure that all ESS (Effective Sample Sizes) were above 200. The maximum credibility tree was inferred by TreeAnnotator 1.5.4 (Drummond and Rambaut 2007) and edited in FigTree 1.4.3. All the phylogenetic trees were rooted with *A. wolterstorffi*.

Results

Sequences spanning 773 bp of the mitochondrial cyt *b* gene, 1141 bp of the mitochondrial CO1 gene and 620 bp of the nuclear ENC1 gene were characterized, respectively, for 93, 102 and 52 individuals of *A. minuano* collected at 6 different localities and 19, 13 and 8 individuals of *A. pongondo* collected at one locality. The *A. minuano* intraspecific matrix encompassed 41 and 47 haplotypes for cyt *b* and CO1, respectively, while the matrix of *A. pongondo* presented 6 and 4 haplotypes, respectively. For ENC1, 41 alleles were detected for *A. minuano* and 11 for *A. pongondo*.

Despite minor differences in variability levels, CO1, cyt *b* and ENC1 presented similar divergence patterns in the target species, and specially the mitochondrial loci subdivided the samples into four distinct clusters: (1) *A. pongondo* (pongondo, with haplotypes sampled at the municipality of Rio Grande – district of Povo Novo, RS, Brazil - located on the West margin of the Patos lagoon); (2) *A. minuano* cluster 1 (minuano 1, clustering haplotypes sampled in seven localities of Rio Grande – district of Ilha do Leonídeo, RS, Brazil - West margin of the Patos lagoon); (3) *A. minuano* cluster 2 (minuano 2, grouping together the haplotypes sampled in three

890 localities of São José do Norte, RS, Brazil (SJN3, SJN4 and SJN/Bujuru) - located on the East
891 margin of the Patos lagoon); and (4) *A. minuano* cluster 3 (minuano 3, with haplotypes sampled
892 at SJN/Bujuru, Tavares and Tavares/Talha Mar, RS, Brazil - located on the East margin of the
893 Patos lagoon).
894
895 **Genetic diversity and population structure**
896 ***Cyt b and CO1 datasets***
897 The haplotype networks reconstructed with cyt *b* and CO1 datasets, both individually or in
898 combination revealed the presence of the four independent haplogroups (Figs. 1B-D): (1)
899 Pongondo; (2) minuano 1; (3) minuano 2; and (4) minuano 3. No haplotype was shared between
900 these haplogroups in any of the three cases (Fig. 1B-D). The population structure that maximized
901 the differentiation between groups while preserving geographical homogeneity in the SAMOVA
902 performed with the mitochondrial concatenated matrix subdivided the samples in three groups
903 (pongondo, minuano 1 and minuano 2 + minuano 3), which were able to explain 61% of the
904 variation encountered. This value rises to 93% when the four clusters are considered. BAPS
905 analysis also suggested subdivision in four clusters.
906 With the concatenated mitochondrial dataset, the number of haplotypes per sampling
907 locality ranged from 4 (minuano 1) to 27 (minuano 3), with minimum Hd of 0.889 and π of 0.00136
908 (pongondo), and maximum Hd and π values of 1.00 and 0.00481 (minuano 2), respectively (Table
909 1). None locality presented significant deviations from neutrality (Table 1). All pairwise
910 comparisons between populations detected significant genetic structure, and Fst values varied from
911 0.86 (in the comparison between minuano 2 and minuano 3) to 0.99 (in the comparison between
912 pongondo and minuano 1) (Table 2). The Mantel test did not indicate a significant correlation
913 between genetic and geographic distances ($r = -0.65$; $p < 1.000$).

Table 1. CO1 + Cyt *b* genetic diversity estimates and neutrality tests for each of the four clusters suggested in this study (see Results).

	CO1 + Cyt *b*					
Groups	**N**	**H**	**Hd**	**π**	**Neutrality tests**	
					Tajima's D	**Fu's F**
Pongondo	9	6	0.889±0.091	0.00136±0.0001	1.7762	-1.496
Minuano 1	5	4	0.900±0.161	0.00178±0.0006	-0.8073	-0.128
Minuano 2	6	6	1.000±0.096	0.00481±0.0008	-0.5443	-1.086
Minuano 3	32	27	0.990±0.010	0.00354±0.0003	-1.4435	-18.444
Total	52	43	0.992±0.005	0.03415±0.0058	-0.1144	-2.329

N, the number of sequences; H, number of haplotypes; Hd, haplotype (gene) diversity; 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 2. CO1 + Cyt *b* pairwise fixation indices (FST) between the four clusters suggested in this study.

Clusters	**Pongondo**	**Minuano 1**	**Minuano 2**	**Minuano 3**
Pongondo	0			
Minuano 1	0.98615*	0		
Minuano 2	0.93882*	0.96557*	0	
Minuano 3	0.92419*	0.96751*	0.85956*	0

Asterisks indicate significant differences ($P < 0.05$).

Net Tamura 3-parameters performed only with cyt *b* sequences revealed that the four lineages differed by a minimum of 0.019 (as seen between populations of minuano 2 and minuano 3) and a maximum of 0.129 (as seen between pongondo and minuano 1) (Table 3, below diagonal).

925 The values for CO1 ranged from 0.020 (as seen between populations of minuano 2 and minuano
926 3) and to 0.108 (as seen between pongondo and minuano 1) (Table 3, above diagonal).
927
928 **Table 3.** Net Tamura 3-parameters distances between the four clusters suggested in this study, as measured by cyt *b*
929 (below diagonal) and COI (above diagonal).

Clusters	Pongondo	Minuano 1	Minuano 2	Minuano 3
Pongondo	0	0.108	0.037	0.032
Minuano 1	0.129	0	0.096	0.098
Minuano 2	0.047	0.116	0	0.020
Minuano 3	0.050	0.123	0.019	0

930
931 ***ENC1 dataset***
932 The haplotype network reconstructed with ENC1 phased alleles somewhat supported
933 subdivision between clusters (Figure 1E), although structure was by no means so clear as that
934 recovered for the mitochondrial loci. In this sense, this network revealed the presence of one
935 putative ancestral allele that is shared between minuano 2 and minuano 3 (Fig. 1E), besides some
936 cases of intermingled structure. Accordingly, population structure suggested by SAMOVA
937 subdivided the samples in three groups (pongondo, minuano 1 and minuano 2 + minuano 3) that
938 were able to explain 51% of the variation encountered. Such a structure was also supported by
939 BAPS.
940 Concerning ENC1 diversity estimates, the number of alleles per sampling locality ranged
941 from 6 (minuano 1) to 19 (minuano 2 and minuano 3), with minimum He of 0.681 and π of 0.0028
942 (minuano 1), and maximum He and π values of 0.942 and 0.00538 (pongondo), respectively (Table
943 4). None locality presented significant deviations from neutrality (Table 4). All pairwise
944 comparisons between populations detected significant genetic structure between clusters, and Fst

values varied from 0.26 (in the comparison between minuano 2 and minuano 3) to 0.79 (in the comparison between minuano 1 and minuano 3) (Table 5). The Mantel test did not indicate a significant correlation between genetic and geographic distances ($r = -0.15$; $p < 0.503$).

Table 4. ENC1 genetic diversity estimates and neutrality tests for each of the four clusters suggested in this study (see Results).

	ENC1					
Groups	**N**	**A**	**He**	**π**	**Neutrality tests**	
					Tajima's D	**Fu's F**
Pongondo	16	11	0.942±0.041	0.00538±0.0010	-1.5368	-4.700
Minuano 1	14	6	0.681±0.132	0.00282±0.0009	-1.4683	-1.333
Minuano 2	38	19	0.939±0.021	0.00466±0.0003	-0.4222	-11.208
Minuano 3	52	19	0.868±0.037	0.00375±0.0005	-1.5040	-11.076
Total	120	52	0.959±0.009	0.00756±0.0004	-1.2814	-43.347

N, the number of sequences; A, number of alleles; 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 5. ENC1 pairwise fixation indices (FST) between the four clusters suggested in this study.

Clusters	**Pongondo**	**Minuano 1**	**Minuano 2**	**Minuano 3**
Pongondo	0			
Minuano 1	0.65396*	0		
Minuano 2	0.31578*	0.69095*	0	
Minuano 3	0.52005*	0.78768*	0.26320*	0

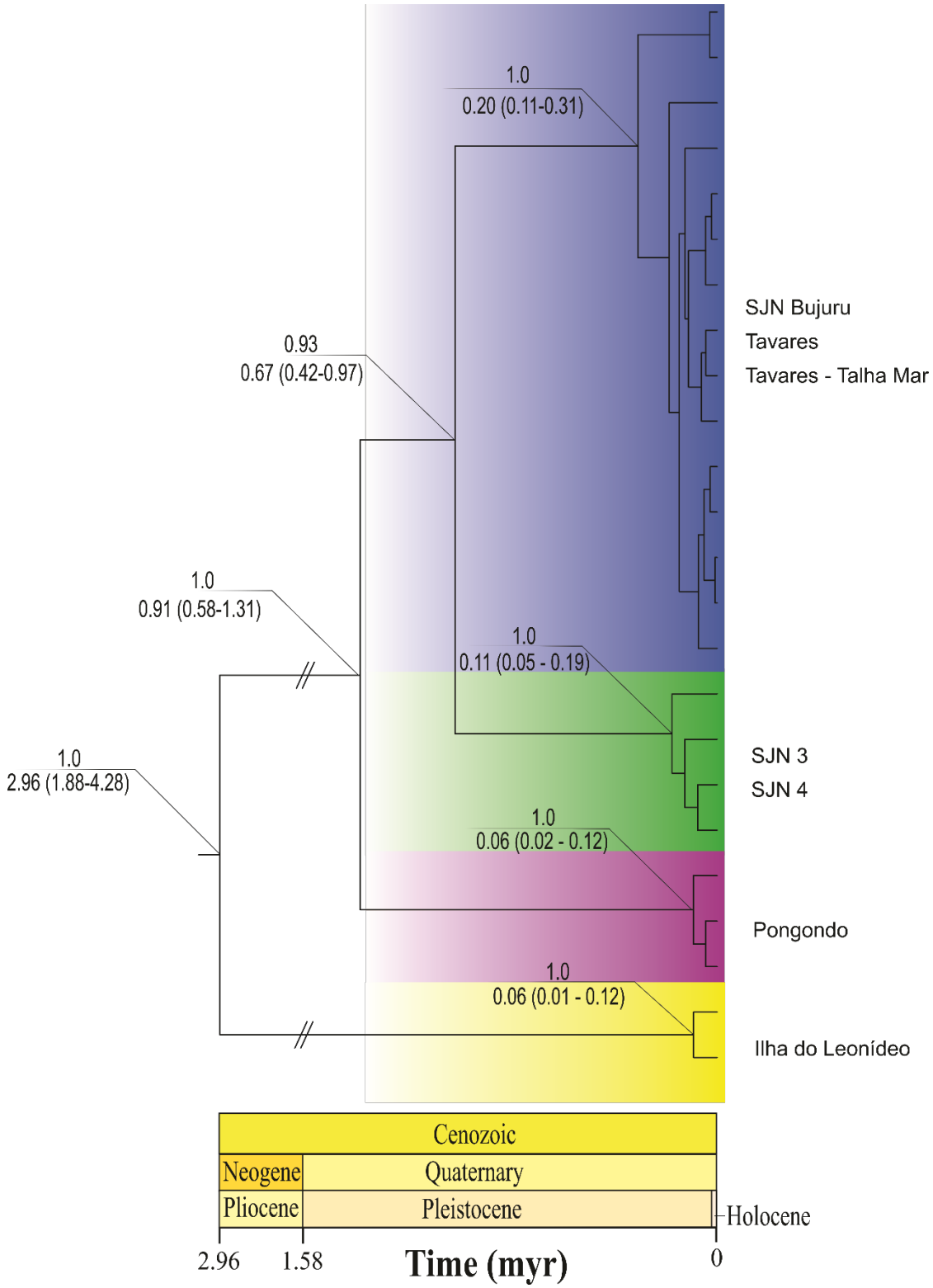
The asterisks indicate significant differences ($P < 0.05$).

957 **Spatiotemporal scenario**

958 **Concatenated dataset**

959 In agreement with previous results, the topologies of the supermatrix trees also supported
960 subdivision into four clusters, which were recovered as reciprocally monophyletic groups in BA
961 tree, with posterior probabilities of 1.00 (Fig. 2). Among the four lineages, minuano 1
962 encompassed the early offshoot, and this divergence was dated to approximately 3 Mya.
963 Conversely, pongondo diverged from the clade encompassing minuano 2 and minuano 3 quite
964 more recently, about 0.9 Mya. Diversifications of each of the four population groups were set
965 between 0.2 and 0.06 myr (Fig. 2).

966



967

968 **Figure 2.** Bayesian chronophylogenetic tree based on cyt *b*, CO1 and ENC1 sequences sampled for *A. minuano* sensu
969 lato e *A. pongondo*. Values above the branches represent support values, given by posterior probabilities values in
970 BA. Values below the branches represent mean and 95% confidence intervals of divergence or diversification dates,
971 as reconstructed in Beast. Groups of populations were represented by their respective names and colors (see other
972 figures).

Discussion

Some species, such as *Austrolebias minuano*, encompass a set of unique populations that are small and isolated from each other by natural or anthropogenic barriers of reproductive isolation (Gibbs 2000). These barriers facilitate the processes of vicariance and allopathic speciation within these environments (Ritchie 2007; Berois et al. 2015). In line with this premise, the analyses revealed that, in addition to several cases of significantly high levels of differentiation between groups of populations, *A. minuano* is subdivided into at least two different population groups, in addition to *A. pongondo*. Such structure was suggested by the Networks, SAMOVA, BAPS and phylogenetic analyses performed individually or in combination for all the employed molecular markers, and, also, supported by the complete absence of mitochondrial haplotype sharing between the groups. This suggests that vicariance has played an important role in the genetic diversification of *A. minuano*, as also revealed for several other species of Rivulidae (Jowers et al. 2008; Bartáková et al. 2013; Ponce de León et al. 2014; García et al. 2015; Loureiro et al. 2015; Garcez et al. 2018). Moreover, once again, genetic data evidenced the role played by large water bodies and large terrestrial areas disproved of suitable pounds as barriers to gene flow in killifish (Maltagliati 1998; Markert et al. 2010).

Allopatric fragmentation seems to be an ongoing process within *A. minuano* and there appears to be no current gene flow between the groups. This can be evidenced through the significant and high FST values recovered between the two groups of populations of *A. minuano* and *A. pongondo*, which differed by a minimum of 4.7% and 3.7% corrected distances regarding the mitochondrial genes cyt *b* and CO1 genes, respectively. In fact, divergence values as high as 12.9% and 10.8% were found for cyt *b* and CO1, respectively, between minuano 1 and *A. pongondo,* two populations that are just 21 km apart and inhabit the west margin of the Patos Lagoon. Thus, the status of *A. pongondo* as a distinct species (Costa et al. 2017) is here corroborated, as well as that of minuano 1, which differed from the other population groups by a

998 minimum of 11.6% for cyt *b* and 9.6% for CO1. As this lineage inhabits the type locality of the
999 species (Costa and Cheffe 2001; Costa et al. 2017) it encompasses the actual *A. minuano*.
1000 Although the east Margin of the Patos Lagoon is clearly inhabited by distinct species of *A.*
1001 *minuano*, the status of the two population groups it embraces is by far not so clear. These
1002 populations encompass reciprocally monophyletic lineages that differ between each other by about
1003 1.9% and 2% for *cytb* and CO1, respectively. As distances as small as 1.4% were previously found
1004 for cyt *b* among different species of *Austrolebias* (Garcia et al. 2000), and even lower ranges (0 to
1005 1.8%) were reported recently within the species complexes of *A. wolterstorffi* and *A. bellottii* (
1006 Steindachner, 1881; García et al. 2015; Alonso et al. 2016; Garcez et al. 2018), we can reliably
1007 state that even minuano 2 and minuano 3 encompass distinct evolutionary lineages, whose
1008 conservation needs to be independently assured. So, this study provides an additional example of
1009 the importance of molecular techniques in recovering the evolutionary status presented by cryptic
1010 populations of killifish, as previously reported by Costa (2013). It is important to emphasize,
1011 nevertheless, that careful morphological inspection needs to be performed in each of these
1012 population groups in order to confirm the level of morphological divergence.
1013 In this scenario where a single species is splitted into a species complex, it is quite
1014 interesting to see that all clusters have shown reasonable levels of genetic diversity. Such results
1015 were previously reported for other species of *Austrolebias* (Garcia et al. 2000; Bartáková et al.
1016 2013; García et al. 2015; Garcez et al. 2018)) and are possibly explained by small-scale events of
1017 gene flow within population groups or even by high mutation rates (García et al. 2015; Garcez et
1018 al. 2018). Nevertheless, this is by no means an indicative that the species are dealing well with
1019 anthropogenic impact. In fact, all the species, or lineages are severely threatened because of their
1020 limited distribution and wide fragmentation and degradation of wetlands in the region (Volcan et
1021 al. 2015).

Concerning the spatio-temporal evolutionary scenario, minuano 1 encompassed the early offshoot of the species complex, being followed by *A. pongondo*. Since these are the lineages encountered on the western margin of the Patos Lagoon, this allows hypothesizing that a colonization event occurred across the Lagoon to form the two clusters found on the eastern margin (minuano 2 and minuano 3). This pattern contrasts with that encountered for *A. wolterstorffi*, for which population groups inhabiting the eastern and western margin of the Patos Lagoon constitute reciprocally monophyletic clusters (Garcez et al. 2018). The contrasting pattern can also be evidenced by the older divergence dates obtained in this study, in which divergence between population groups were dated between 3-0.67 Mya. This suggests ancestral populations of the *A. minuano* complex were diversifying quite earlier than the occurrence of the first Pleistocene Lagoon Barrier Depositional System related to the paleogeographic evolution of the South American Coastal Plain, dated to approximately 400 kyr (Montaña and Bossi 1995; Tomazelli and Villwock 2005; Villwock and Tomazelli 2007). This pattern will certainly be complemented by further inclusion of samples from the other species of the *A. adloffi* species group, since there are possibly more intermediate clades to be added in this picture.

In general, this study certainly helps in promoting a better comprehension of the evolution and conservation concerns related to the diversity of annual fishes (which include one of the most threatened vertebrate groups in the country) (ICMbio 2012; Volcan et al. 2015) on the South American Coastal Plain. It is hopeful, therefore, that the knowledge achieved here will be applied in the implementation and promotion of environmental education programs, thus encouraging the demarcation of new protection areas. Only a set of actions can save this "unique and biologically fascinating group of species" (Garcez et al. 2018) from extinction.

Conflict of interest

There is no conflict of interest.

All authors contributed equally to this work.

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1222 **Supplementary Material**

1223

1224 **Table S 1.** Summary of sampling localities, with their respective identification codes, geographical coordinates and

1225 amplified genes

		General Information		Amplified Genes		
Species	**ID**	**Population**	**Coordinates**	**CO1**	**Cyt *b***	**ENC1**
A. pongongo	1M1	RG - Povo Novo	-31.922537 -52.312918		X	X
A. pongongo	1M2	RG - Povo Novo	-31.922537 -52.312918			
A. pongongo	1M3	RG - Povo Novo	-31.922537 -52.312918		X	
A. pongongo	1M4	RG - Povo Novo	-31.922537 -52.312918	X	X	
A. pongongo	1M5	RG - Povo Novo	-31.922537 -52.312918	X	X	
A. pongongo	1M6	RG - Povo Novo	-31.922537 -52.312918	X	X	
A. pongongo	1M7	RG - Povo Novo	-31.922537 -52.312918	X	X	X
A. pongongo	1M8	RG - Povo Novo	-31.922537 -52.312918	X	X	
A. pongongo	1M9	RG - Povo Novo	-31.922537 -52.312918		X	
A. pongongo	1M10	RG - Povo Novo	-31.922537 -52.312918	X	X	
A. pongongo	1M11	RG - Povo Novo	-31.922537 -52.312918	X	X	X
A. pongongo	1M12	RG - Povo Novo	-31.922537 -52.312918	X	X	X
A. pongongo	1M13	RG - Povo Novo	-31.922537 -52.312918	X	X	X
A. pongongo	1M14	RG - Povo Novo	-31.922537 -52.312918	X	X	
A. pongongo	1M15	RG - Povo Novo	-31.922537 -52.312918		X	X
A. pongongo	1M16	RG - Povo Novo	-31.922537 -52.312918		X	X
A. pongongo	1M17	RG - Povo Novo	-31.922537 -52.312918	X	X	
A. pongongo	1M18	RG - Povo Novo	-31.922537 -52.312918	X	X	
A. pongongo	1M19	RG - Povo Novo	-31.922537 -52.312918		X	X
A. pongongo	1M20	RG - Povo Novo	-31.922537 -52.312918	X	X	
A. minuano	2M1	RG - Ilha do Leonídeo	-32.050478 -52.227949	X	X	
A. minuano	2M2	RG - Ilha do Leonídeo	-32.050478 -52.227949			
A. minuano	2M3	RG - Ilha do Leonídeo	-32.050478 -52.227949	X	X	X
A. minuano	2M4	RG - Ilha do Leonídeo	-32.050478 -52.227949		X	
A. minuano	2M5	RG - Ilha do Leonídeo	-32.050478 -52.227949	X		
A. minuano	2M6	RG - Ilha do Leonídeo	-32.050478 -52.227949		X	
A. minuano	2M7	RG - Ilha do Leonídeo	-32.050478 -52.227949		X	
A. minuano	2M8	RG - Ilha do Leonídeo	-32.050478 -52.227949	X	X	
A. minuano	2M9	RG - Ilha do Leonídeo	-32.050478 -52.227949			
A. minuano	2M10	RG - Ilha do Leonídeo	-32.050478 -52.227949		X	X
A. minuano	2M11	RG - Ilha do Leonídeo	-32.050478 -52.227949		X	X
A. minuano	2M12	RG - Ilha do Leonídeo	-32.050478 -52.227949	X		
A. minuano	2M13	RG - Ilha do Leonídeo	-32.050478 -52.227949	X		
A. minuano	2M14	RG - Ilha do Leonídeo	-32.050478 -52.227949	X	X	
A. minuano	2M15	RG - Ilha do Leonídeo	-32.050478 -52.227949	X	X	X

A. minuano	2M16	RG - Ilha do Leonídeo	-32.050478 -52.227949	X	X	X
A. minuano	2M17	RG - Ilha do Leonídeo	-32.050478 -52.227949		X	X
A. minuano	2M18	RG - Ilha do Leonídeo	-32.050478 -52.227949		X	X
A. minuano	2M19	RG - Ilha do Leonídeo	-32.050478 -52.227949	X	X	
A. minuano	2M20	RG - Ilha do Leonídeo	-32.050478 -52.227949		X	
A. minuano	3M1	SJN - 3	-31.9172 -51.94305			
A. minuano	3M2	SJN - 3	-31.9172 -51.94305	X		X
A. minuano	3M3	SJN - 3	-31.9172 -51.94305	X		
A. minuano	3M4	SJN - 3	-31.9172 -51.94305	X		X
A. minuano	3M5	SJN - 3	-31.9172 -51.94305	X		X
A. minuano	3M6	SJN - 3	-31.9172 -51.94305	X		X
A. minuano	3M7	SJN - 3	-31.9172 -51.94305	X		X
A. minuano	3M8	SJN - 3	-31.9172 -51.94305	X		X
A. minuano	3M9	SJN - 3	-31.9172 -51.94305	X		X
A. minuano	3M10	SJN - 3	-31.9172 -51.94305	X		X
A. minuano	3M11	SJN - 3	-31.9172 -51.94305	X		
A. minuano	3M12	SJN - 3	-31.9172 -51.94305	X		X
A. minuano	3M13	SJN - 3	-31.9172 -51.94305	X		
A. minuano	3M14	SJN - 3	-31.9172 -51.94305	X		X
A. minuano	3M15	SJN - 3	-31.9172 -51.94305	X	X	
A. minuano	3M16	SJN - 3	-31.9172 -51.94305		X	
A. minuano	3M17	SJN - 3	-31.9172 -51.94305	X		
A. minuano	3M18	SJN - 3	-31.9172 -51.94305	X	X	X
A. minuano	3M19	SJN - 3	-31.9172 -51.94305	X	X	
A. minuano	3M20	SJN - 3	-31.9172 -51.94305	X		
A. minuano	4M1	SJN - 4	-31.88016 -51.862027	X		
A. minuano	4M2	SJN - 4	-31.88016 -51.862027	X		
A. minuano	4M3	SJN - 4	-31.88016 -51.862027	X		
A. minuano	4M4	SJN - 4	-31.88016 -51.862027	X		
A. minuano	4M5	SJN - 4	-31.88016 -51.862027	X	X	X
A. minuano	4M6	SJN - 4	-31.88016 -51.862027	X		
A. minuano	4M7	SJN - 4	-31.88016 -51.862027	X		X
A. minuano	4M8	SJN - 4	-31.88016 -51.862027	X		X
A. minuano	4M9	SJN - 4	-31.88016 -51.862027	X		
A. minuano	4M10	SJN - 4	-31.88016 -51.862027	X		X
A. minuano	4M11	SJN - 4	-31.88016 -51.862027	X	X	X
A. minuano	4M12	SJN - 4	-31.88016 -51.862027	X		
A. minuano	4M13	SJN - 4	-31.88016 -51.862027	X	X	
A. minuano	4M14	SJN - 4	-31.88016 -51.862027	X		
A. minuano	4M15	SJN - 4	-31.88016 -51.862027	X		
A. minuano	4M16	SJN - 4	-31.88016 -51.862027	X	X	X
A. minuano	4M17	SJN - 4	-31.88016 -51.862027	X		
A. minuano	4M18	SJN - 4	-31.88016 -51.862027	X		
A. minuano	4M19	SJN - 4	-31.88016 -51.862027	X		

A. minuano	4M20	SJN - 4	-31.88016 -51.862027	X		X
A. minuano	5M1	SJN - Bujuru	-31.5272 -51.291	X		X
A. minuano	5M2	SJN - Bujuru	-31.5272 -51.291	X	X	
A. minuano	5M3	SJN - Bujuru	-31.5272 -51.291	X		
A. minuano	5M4	SJN - Bujuru	-31.5272 -51.291	X	X	
A. minuano	5M5	SJN - Bujuru	-31.5272 -51.291	X		X
A. minuano	5M6	SJN - Bujuru	-31.5272 -51.291	X	X	X
A. minuano	5M7	SJN - Bujuru	-31.5272 -51.291	X	X	X
A. minuano	5M8	SJN - Bujuru	-31.5272 -51.291	X	X	
A. minuano	5M9	SJN - Bujuru	-31.5272 -51.291	X	X	
A. minuano	5M10	SJN - Bujuru	-31.5272 -51.291	X	X	X
A. minuano	5M11	SJN - Bujuru	-31.5272 -51.291			
A. minuano	5M12	SJN - Bujuru	-31.5272 -51.291	X	X	
A. minuano	5M13	SJN - Bujuru	-31.5272 -51.291	X	X	
A. minuano	5M14	SJN - Bujuru	-31.5272 -51.291		X	
A. minuano	5M15	SJN - Bujuru	-31.5272 -51.291	X		
A. minuano	5M16	SJN - Bujuru	-31.5272 -51.291	X	X	
A. minuano	5M17	SJN - Bujuru	-31.5272 -51.291	X		X
A. minuano	5M18	SJN - Bujuru	-31.5272 -51.291	X	X	X
A. minuano	5M19	SJN - Bujuru	-31.5272 -51.291	X	X	
A. minuano	5M20	SJN - Bujuru	-31.5272 -51.291	X	X	
A. minuano	6M1	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M2	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M3	Tavares	-31.291687 -51.081523	X	X	X
A. minuano	6M4	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M5	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M6	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M7	Tavares	-31.291687 -51.081523	X	X	X
A. minuano	6M8	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M9	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M10	Tavares	-31.291687 -51.081523	X	X	X
A. minuano	6M11	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M12	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M13	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M14	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M15	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M16	Tavares	-31.291687 -51.081523	X	X	X
A. minuano	6M17	Tavares	-31.291687 -51.081523		X	
A. minuano	6M18	Tavares	-31.291687 -51.081523	X	X	
A. minuano	6M19	Tavares	-31.291687 -51.081523	X	X	X
A. minuano	6M20	Tavares	-31.291687 -51.081523		X	
A. minuano	7M1	Tavares - Talha Mar	-31.240750 -51.004880	X		X
A. minuano	7M2	Tavares - Talha Mar	-31.240750 -51.004880	X		
A. minuano	7M3	Tavares - Talha Mar	-31.240750 -51.004880	X	X	X

A. minuano	7M4	Tavares - Talha Mar	-31.240750 -51.004880	X	X	X
A. minuano	7M5	Tavares - Talha Mar	-31.240750 -51.004880	X		X
A. minuano	7M6	Tavares - Talha Mar	-31.240750 -51.004880	X		X
A. minuano	7M7	Tavares - Talha Mar	-31.240750 -51.004880	X	X	
A. minuano	7M8	Tavares - Talha Mar	-31.240750 -51.004880			X
A. minuano	7M9	Tavares - Talha Mar	-31.240750 -51.004880	X		X
A. minuano	7M10	Tavares - Talha Mar	-31.240750 -51.004880	X	X	X
A. minuano	7M11	Tavares - Talha Mar	-31.240750 -51.004880	X	X	X
A. minuano	7M12	Tavares - Talha Mar	-31.240750 -51.004880			
A. minuano	7M13	Tavares - Talha Mar	-31.240750 -51.004880	X		X
A. minuano	7M14	Tavares - Talha Mar	-31.240750 -51.004880	X	X	X
A. minuano	7M15	Tavares - Talha Mar	-31.240750 -51.004880	X	X	X
A. minuano	7M16	Tavares - Talha Mar	-31.240750 -51.004880	X		X
A. minuano	7M17	Tavares - Talha Mar	-31.240750 -51.004880	X	X	
A. minuano	7M18	Tavares - Talha Mar	-31.240750 -51.004880	X	X	X
A. minuano	7M19	Tavares - Talha Mar	-31.240750 -51.004880	X	X	X
A. minuano	7M20	Tavares - Talha Mar	-31.240750 -51.004880	X	X	

1226 The initials RG mean Rio Grande and SJN means São José do Norte.

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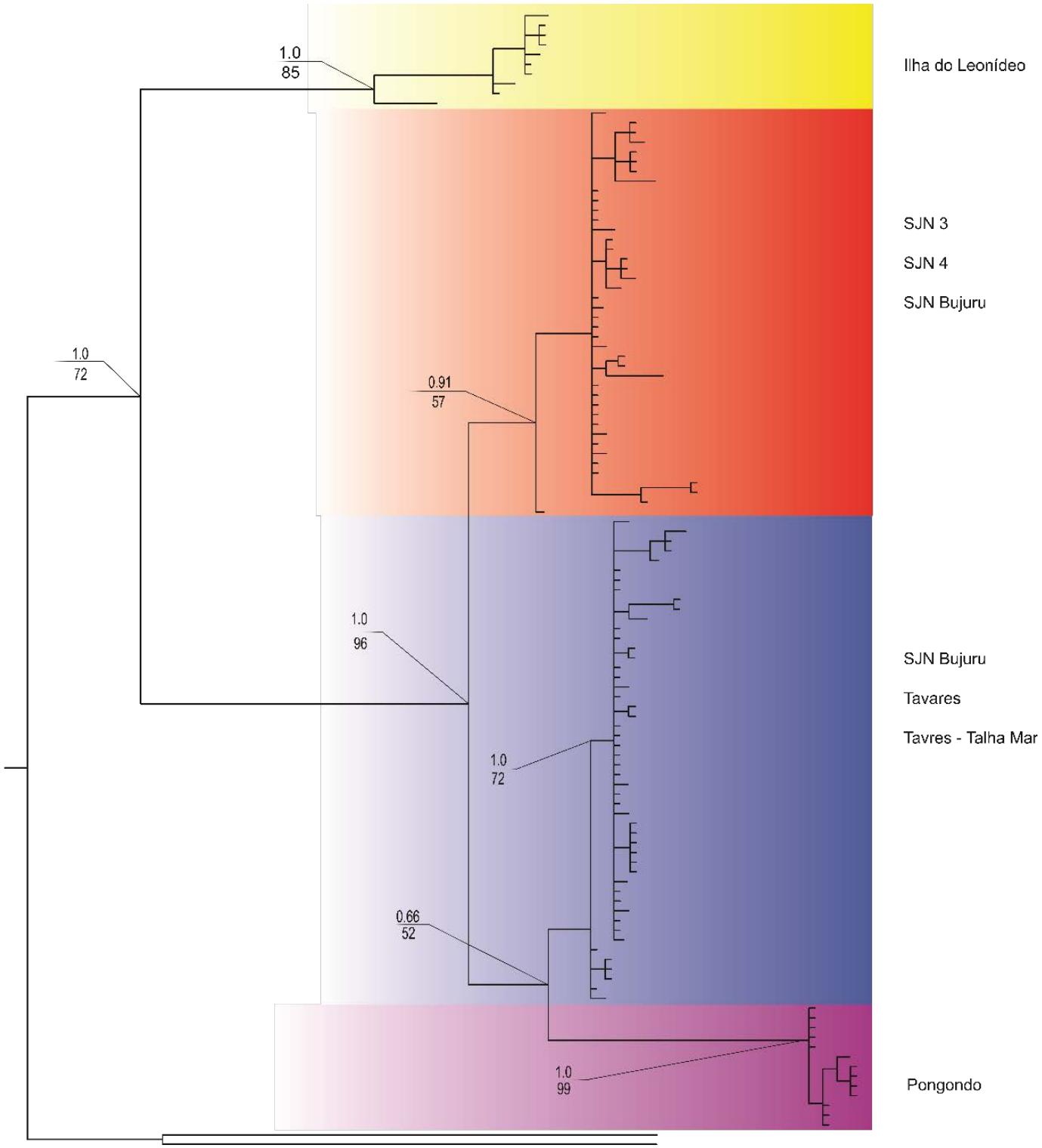
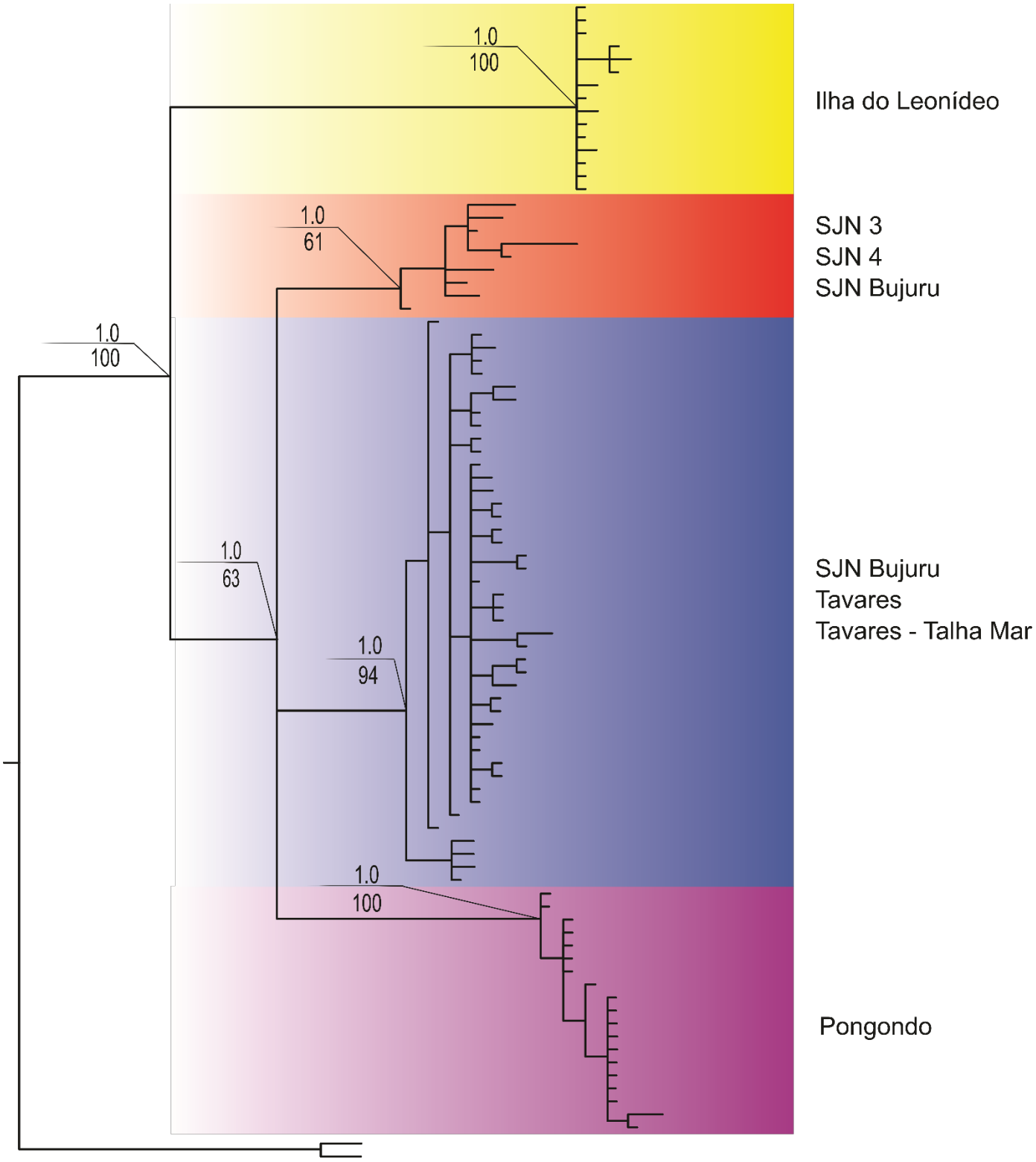


Figure S 1. Phylogenetic tree generated from the amplification of the mitochondrial CO1 gene in *Austrolebias minuano*



1232

1233 **Figure S 2.** Phylogenetic tree generated from the amplification of the mitochondrial cyt *b* gene in *Austrolebias*

1234 *minuano*

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CAPÍTULO 2

Artigo a ser submetido na revista “Aquaculture Research”

Dimethylsulfoxide, glycerol and methylglycol in the sperm cryopreservation of annual fish *Austrolebias minuano* (Cyprinodontiformes: Rivulidae)

Sperm freezing of *Austrolebias minuano*

Murilo de Oliveira Fernandes[1,2] (https://orcid.org/0000-0003-2030-4119), Daiana Kaster Garcez[1,2] (https://orcid.org/0000-0003-4526-9647), Izani Bonel Acosta[2,3], Stela Mari Meneguello[2,3], Carine Dahl Corcini[2,3] (https://orcid.org/0000-0001-5683-7801), Lizandra Jaqueline Robe[1,4] (https://orcid.org/0000-0001-8506-9143), Antonio Sergio Varela Junior[2,3] (https://orcid.org/0000-0003-4901-5118)

[1]Programa de Pós-Graduação em Biologia de Ambientes Aquáticos Continentais (PPGBAC), Universidade Federal do Rio Grande (FURG), Rio Grande 96203-000, Rio Grande do Sul, Brazil.

[2]Reprodução Animal Comparada, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande (FURG), Rio Grande, Rio Grande do Sul, Brazil.

[3]Centro de Pesquisa e Educação em Reprodução Animal, Faculdade de Veterinária, Universidade Federal de Pelotas (UFPel), Pelotas, Brazil.

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

* Corresponding author: murilo_fernan@hotmail.com (MOF)

All authors contributed equally to this work.

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ABSTRACT

Austrolebias minuano is an annual Brazilian fish threatened due to loss and degradation of its habitat. Therefore, the use of techniques, such as cryopreservation, allow the storage of at least part of its genetic heritage. The objective of this study was to evaluate the effects of different cryoprotectants (dimethylsulfoxide, glycerol and methyl glycol) in different concentrations on the fish sperm quality parameters of *Austrolebias minuano*. Male sperm was used for evaluations of cell kinetic parameters and flow cytometry. In this study, the best results regarding the parameters of sperm kinetics were presented in cryopreserved samples with 7.5% methyl glycol. Instead, dimethylsulfoxide at its concentration of 7.5% and glycerol at concentrations of 10, 12.5 and 15% were the least effective treatments for all parameters evaluated for spermatic kinetics. Likewise, for flow cytometry, the concentration of 7.5% methyl glycol was efficient and dimethylsulfoxide was less efficient in the concentration of 12.5% in relation to the several parameters evaluated. Therefore, we concluded that for the preservation of sperm quality in *A. Minuano*, the use of cryoprotectants, specially methyl glycol, is effective. However, it was found that there is a high sensitivity of these spermatozoa to glycerol; which is not recommended for preservation purposes.

KEYWORDS

cryoprotectants, extinction, killifish, preservation

1 INTRODUCTION

In recent decades, human action has devastated more natural areas than all of humanity in thousands of years (Koneswaran & Nierenberg, 2008). The consequences of habitat loss and extinction of certain populations or species may affect an entire community and severely impair their diversity (Humphries & Winemiller, 2009). This effect is particularly severe in continental aquatic environments where small species are more vulnerable to extinction (Olden, Hogan, & Zanden, 2007).

Composing a large part of the aquatic biodiversity, annual fish belonging to the Rivulidae family, representing 30% of the ichthyofauna threatened in Brazil (ICMbio, 2012). The threat faced by the group is mainly due to the loss and fragmentation of temporary continental aquatic environments, which has been particularly drastic (Fontana, Bencke, & Reis, 2003). The fact that they are small and isolated populations makes the group especially susceptible to high rates of genetic drift, accelerating evolutionary rates, and promoting the occurrence of allopatric speciation events (Beheregaray, Attard, Brauer, & Hammer, 2016). This further intensifies the threat status of the different groups and evidences the need for immediate conservation of the genetic heritage of the different lineages.

Austrolebias minuano (Costa & Cheffe, 2001) (Fig. 1) is one of the species of annual fishes threatened of extinction in Brazil (Rosa & Lima, 2008). This species belongs to the group *A. adloffi*, distinguished by its body size and color patterns. Within the distribution perspectives of the different species of annual fish, *A. minuano* presents a relatively wide distribution, occurring in ponds of the Patos Mirim lagoon system (Costa, 2006). Its opportunistic food habit makes it particularly important for food chains and the balance of wetland ecosystems (Volcan, Fonseca, & Robaldo, 2011).

Ex situ conservation strategies allow the creation of germplasm banks, a technique that ensures the preservation of at least part of the genetic heritage of endangered species (Figueroa et al., 2015; Silva et al., 2016). The availability of genetic resources, such as spermatozoa and cryopreserved oocytes, allows the recovery of these species, avoiding the loss of genetic diversity, and enabling the

reintroduction into the natural environment. Hence, this technique provides a means to recover and preserve endangered species through the conservation of biodiversity (Cabrita et al., 2010; Martínez-Páramo et al., 2009).

Fig. 1. Species of *Austrolebias minuano*, withdrawal from the locality type Ilha do Leonídeo, state of Rio Grande do Sul - Brazil. The male of the species (A) is characterized by pairs of dark lines in its body and the female (B) for its smooth lines in the body.

However, as the biochemical composition of sperm varies widely, it is necessary to develop a cryopreservation protocol for every species (Carolsfeld, Godinho, Zaniboni Filho, & Harvey, 2003; Shin, Lein, Patten, & Ruhnke, 1988). To establish an efficient and practical protocol for sperm cryopreservation, factors such as cryoprotectant type and concentration, conditions for freezing and thawing of sperm, diluents and time of each process should be standardized. According to Viveiros et al., (2012) the intracellular cryoprotectants most used for cryopreservation of fish are dimethyl

sulfoxide (DMSO), glycerol and methyl glycol. In fact, cryoprotectants of the alcohol class have been widely used in sperm freezing in freshwater fish species: Pirapitinga (*Brycon nattereri*) (Oliveira, Viveiros, Maria, Freitas, & Izaú, 2007), Tambaqui (*Colossoma macropomum*) (Menezes, Queiroz, Doria, & Menezes Jr, 2008; Varela Junior et al., 2012), Curimba (*Prochilodus lineatus*) (Viveiros & Godinho, 2009).

Dimethyl sulfoxide has confirmed significant results in the semen freezing of fish because it is a highly soluble element in the aqueous medium that easily penetrates the plasma membrane of the cell, binding to the free hydroxyl responsible for the formation of reactive oxygen species (MacGregor, 1967). Another alcohol widely used in programs for the preservation of male gametes of several species is glycerol (Alvarenga, Papa, Landim-Alvarenga, & Medeiros, 2005). However, this component has shown some disadvantages when compared to other cryoprotectants, especially on the integrity of cellular structures, due to its high molecular weight, high viscosity and high level of impermeability (Hammerstedt, Graham, & Carolina, 1992). Finally, different studies indicate that methyl glycol is ideal for the preservation of fish spermatozoa as *Brycon insignis* (Viveiros et al., 2011), *B. orbignyanus* (Maria, Viveiros, Freitas, & Oliveira, 2006), *Piaractus brachypomus* (Nascimento, Maria, Pessoa, Carvalho, & Viveiros, 2010), *Piaractus mesopotamicus* (Órfão, L.H., Viveiros, A.T.M., Silva, F.P.C., Maria, 2008), *P. lineatus* (Viveiros, Maria, Orfao, Carvalho, & Nunes, 2008) and *Colossoma macropomun* (Carneiro, Azevedo, Santos, & Maria, 2012).

In this context, the objective of the present study was to standardize the first cryopreservation protocol for an annual fish species of the Rivulidae family.

2 MATERIALS AND METHODS

2.1 Animals and semen collection

Adult males of *Austrolebias minuano* were collected with the aid of immersion nets in the type locality of the species (Costa, 2006), located on Ilha do Leonídeo (52°22'79.49"S and 32°05'04.78"W), in Rio

Grande, RS, southern Brazil. The animals were euthanized by a section of the spinal cord, a method accepted with restrictions for laboratory fish, according to the Federal Council of Veterinary Medicine of Brazil (Resolution No. 1000 of 05/2012), when the use of anesthetics may affect the results of the analyzes.

In this study, with the objective of increasing the volume of semen per sample, a pool of gonads of three males was carried out for each of the 15 samples, totaling 45 animals. After collection, the semen was diluted in Beltsville Thawing Solution (BTS), pH 7.2 and osmolarity 380 mOsm / kg in a ratio of 1: 9 (v / v). For the estimation of motility, 1 µl of semen diluted in 4 µl of water (from the place where they were collected) and analyzed by means of sperm analysis aided by Computer Assisted Semen Analysis (CASA) (Dziewulska, Rzemieniecki, Czerniawski, & Domagała, 2011). All samples used in the experiment did not have total motility prior to activation. On the other hand, after 10 seconds of the activation, all had motility superior to 80%, without contamination by urine or feces.

2.1.2 Experimental design

The freezing counted on 15 pools distributed in 15 treatments using the cryoprotectants DMSO, Methyl glycol and Glycerol in the concentrations of 5; 7.5; 10; 12.5 and 15%. All treatments were prepared in BTS® (Beltsville Thawing Solution) diluent (Pursel & Johnson, 1975) with pH of 7.2 and osmolarity of 380 mOsm. Each treatment was placed in 250 µL containers, sealed with polyvinyl alcohol, placed in metal racks and kept cooled at 5 ° C for 20 minutes. After the exposure time between sperm and treatment, the racks were transferred to the canister of the dry shipper nitrogen vapor boiler (Taylor Wharton, model CP 300), where they remained for at least 12 hours. Then, they were transferred to the liquid nitrogen canister (MVE, model CP-34), where they remained for 45 days until the moment of thawing for analysis of the samples.

2.2 Analysis of spermatic kinetics

The spermatozoa were activated with 1 µL of sample and 4 µL of puddle water, filtered in Millipore (Millex® JBR610021 / 0,22 µm) with the parameters recorded by CASA. Ten fields were captured, having at least 1,000 cells at the end. The parameters evaluated were: Total Motility (MT-%), Progressive Motility (MP%), Average Distance Traveled (DAP-µm), Curvilinear Distance (DCL-µm), VAP-%), Rectilinearity (STR-%), Linearity (LIN-%), Oscillation (WOB), Head Lateral Displacement (ALH-µm) and Cross-Flagellar Beating Frequency (BCF-Hz). The motile period was evaluated from the moment of activation until the stop of the progressive movement of spermatozoa (Varela Junior et al., 2015).

2.3 Flow cytometry analyses

All evaluations were performed in the Attune Acoustic Focusing® version 2.1 (Life Technologies), equipped with a blue laser (Argon 488 nm) and violet laser (UV 405 nm), is the last used to detect all the evaluations (450/40, VL -1). Sperm cell populations were stained with Hoechst 33342 at 16.2 mM concentration, except for DNA fragmentation (Martinez-Alborcia et al., 2012). Non-sperm events were discarded by FSC x SSC and Hoechst 33342 negative (debris) scatter plots. For reading all parameters, cells stained with fluorophores were added in calcium-free PBS (80g NaCl, 11.5 g KCl, 24 g Na 2 HPO 4, 2 g KH 2 PO 4 in 1L deionized water) using a total of 10,000 sperm per analysis.

2.3.1 Mitochondrial Functionality

To assess mitochondrial functionality, 10 µL of thawed sample was incubated with 3.1 µM Rhodamine 123 (green fluorescence) and 7.5 µM IP for 10 min. Sperm cells were classified as having either high functionality (high fluorescence by Rhodamine accumulation) or low functionality (low fluorescence, low Rhodamine accumulation) after they had been analyzed for the presence of intact spermatozoa (IP negative) (Liu et al., 2015). The mitochondrial functionality

rate was calculated by the following formula: [(number of spermatozoa with high mitochondrial membrane potential) / (high sperm count potential of mitochondrial membrane + spermatozoa with low mitochondrial membrane potential)] × 100 (Alves et al., 2016).

2.3.2 Membrane functionality and Cellular disruption

The membrane functionality was verified through the fluorophores Sybr14 and propidium iodide (PI) (Minitübe, Tiefenbach, Germany). The aliquots of thawed semen were mixed with the fluorescent probes containing 0.25 μM of Sybr14 and 7.5 μM IP (as instructed by the manufacturer - Minitube) and incubated for 5 minutes. Functional membrane spermatozoa (Sybr + / IP-) were classified as undamaged, while the others (Sybr + / IP +, Sybr- / IP +, Sybr- / IP-) were classified as lesions (Figueroa et al., 2015). To verify the percentage of cellular rupture, the cells that were IP − were classified as non–ruptured whereas those that were IP + were regarded as ruptured.

2.3.3 Fluidity membrane

The membrane fluidity check was performed using 2.7 μM of hydrophobic merocyanine dye 540 (M540) and 0.1 μM of YO PRO-1 (Invitrogen-Eugene, OR, USA) in 10 μl of thawed sample, for 5 min. High fluidity (high concentration of M540) and low fluidity (low concentration of M540) cells were evaluated only for intact spermatozoa (YO-PRO negative) (Fernández-Gago, Domínguez, & Martínez-Pastor, 2013). The membrane flow rate was calculated by the number of spermatozoa with high fluidity/number of spermatozoa with low fluidity + spermatozoa with high fluidity *100.

2.3.4 DNA fragmentation index

DNA integrity was assessed by the chromatin structure assay (SCSA). To verify this parameter, 10 μL of thawed spermatozoa were added to 5 μL of TNE (0.01 M Tris-HCl, 0.15 M NaCl, 0.001

M EDTA, pH 7.2) and 10 µL of 1X Triton (Triton X-100, 1% (v / v) at 30-second intervals. The acridine orange dye was added and incubated for 30 seconds, not exceeding the time of 2 min for the reading. Spermatozoa were classified as integers (green) or fragmented (orange/red) (Jenkins, Draugelis-Dale, Pinkney, Iwanowicz, & Blazer, 2015). The rate of DNA fragmentation was calculated by the number of spermatozoa with fragmented DNA / sperm count intact DNA + spermatozoa with fragmented DNA *100.

2.3.5 Concentration of reactive oxygen species (ROS)

The concentration of ROS in sperm cells was performed by 1.0 µM of the fluorophore 2'7' dichlorofluorescein diacetate (H2DCFDA) (emits green fluorescence when oxidized) and IP 7.5 µM. The median intensity of green fluorescence was used only for live sperm (IP-) (Domínguez-Rebolledo et al., 2011).

2.3.6 Lipid peroxidation (LPO)

Lipid peroxidation of spermatozoa was evaluated shortly after thawing. The final concentration of 1 µM Bodipy C11 (Hagedorn, McCarthy, Carter, & Meyers, 2012) in 10 µL of sample, and these were incubated for 2 hours at room temperature (20 ° C), and only live spermatozoa were analyzed. The lipid peroxidation rate was calculated by the median intensity of red fluorescence (non-peroxidized lipid) / median green fluorescence intensity + median red fluorescence intensity *100.

2.4 Statistical Analyzes

The variables were evaluated for normality by the Shapiro-Wilk test, followed by analysis of variance (ANOVA) and Tukey's test. The different cryoprotectants and their concentrations were considered independent variables and all other variables such as total motility, progressive motility, DAP, DCL, DSL, VAP, STR, LIN, WOB, ALH, BCF, mitochondrial functionality,

membrane functionality, cell disruption, membrane fluidity, DNA fragmentation, ROS e LPO were considered dependent variables. All analyzes were performed in Statistix® 9.0 software (Statistix, 2008). Values of $P < 0.05$ were considered to indicate significant differences.

3 RESULTS

The best results regarding the parameters of sperm kinetics were presented in cryopreserved samples with methyl glycol (MG) at a concentration of 7.5% ($P < 0.05$), with a progressive motile value of 9.62 ± 0.92 (Table 1). On the other hand, DMSO at its concentration of 7.5% and GLY at concentrations of 10, 12.5 and 15% were the least effective treatments for all parameters evaluated for sperm kinetics (Table 1).

Table - 1. Analyses of total motility, progressive motility, mean distance traveled, curvilinear distance, rectilinear distance, lateral head displacement and frequency of cross flagellar beating of *Austrolebias minuano* (Costa & Cheffe, 2001), with dimethylsulfoxide, glycerol and methyl glycol alcohols at different concentrations (mean ± standard error)

	Spermatic Kinetics						
Treatments (%)	**MT**	**MP**	**DAP**	**DCL**	**DSL**	**ALH**	**BCF**
DMSO 5	8.41±0.9$^{bc\ def}$	4.64±0.7^{cd}	5.69±0.7$^{e\ fg}$	7.46±0.9^{de}	4.41±0.6^{ef}	0.88±0.1$^{ab\ cde}$	10.71±1.1^{def}
DMSO 7.5	7.22±0.9$^{de\ f}$	3.22±0.7^{d}	3.96±0.6$^{g\ hij}$	5.38±0.7$^{e\ fg}$	3.30±0.5^{fgh}	0.69±0.1$^{cd\ efg}$	8.32±1.1^{fg}
DMSO 10	11.13±0.9^{ab}	7.27±0.8^{b}	7.81±0.6$^{b\ cd}$	10.86±0.9^{bc}	6.20±0.5^{bc}	0.81±0.1$^{bc\ def}$	13.61±1.1^{bcd}
DMSO 12.5	7.36±0.6$^{bc\ def}$	3.55±0.4^{d}	5.20±0.5$^{f\ gh}$	7.44±0.7^{de}	3.66±0.3^{fg}	0.65±0.1$^{de\ fg}$	10.83±1.1^{def}
DMSO 15	9.94±0.9$^{ab\ cd}$	5.77±0.8^{bc}	6.05±0.6$^{d\ ef}$	8.81±0.9$^{c\ d}$	4.48±0.4^{def}	0.85±0.1$^{ab\ cdef}$	10.06±1.0^{ef}
GLY 5	5.86±0.9^{f}	3.48±0.7^{d}	4.91±0.6$^{f\ ghi}$	6.83±0.8^{def}	3.80±0.4^{fg}	0.63±0.1$^{de\ fg}$	11.03±1.3^{cdef}
GLY 7.5	8.73±0.9$^{bc\ de}$	4.74±0.7^{cd}	7.20±0.9$^{c\ de}$	10.24±1.1^{bc}	5.89±0.8^{bcd}	1.00±0.1^{ab}	12.12±1.1^{bcde}
GLY 10	5.97±0.8^{ef}	2.80±0.7^{d}	3.38±0.4^{ij}	4.54±0.5$^{f\ g}$	2.61±0.3^{gh}	0.61±0.1$^{ef\ g}$	8.37±1.1^{fg}
GLY 12.5	6.20±0.7$^{de\ f}$	3.20±0.5^{d}	3.51±0.4$^{h\ ij}$	4.87±0.6$^{f\ g}$	2.63±0.3^{gh}	0.66±0.1$^{de\ f}$	6.43±0.9^{g}
GLY 15	6.46±0.8$^{de\ f}$	2.69±0.5^{d}	2.71±0.4^{j}	4.01±0.6^{g}	2.01±0.3^{h}	0.60±0.1^{fg}	6.37±0.9^{g}

MG 5	7.97±0.8^{cd} ef	4.23±0.7^{cd}	4.41±0.6^{f} ghij	5.88±0.8^{e} fg	3.69±0.5 fg	0.53±0.1^{g}	8.56±1.1^{fg}
MG 7.5	14.45±1.8 a	9.62±0.9^{a}	10.28±0.7^{a}	14.63±1.0^{a}	7.84±0.5 a	1.11±0.1^{a}	16.86±1.1 a
MG 10	11.42±0.8 ab	7.43±0.7^{b}	9.55±0.7^{a} b	14.96±1.2^{a}	6.96±0.5 ab	1.05±0.1^{ab}	17.10±1.2 a
MG 12.5	11.24±0.9 ab	6.85±0.7^{b}	7.14±0.5^{c} de	10.58±0.8^{bc}	5.31±0.4 cde	0.90±0.1^{ab} cd	14.06±1.1 abc
MG 15	10.91±0.9 abcd	7.36±0.8^{b}	8.04±0.6^{b} c	11.58±0.9^{b}	5.99±0.4 bc	0.94±0.1^{ab} c	14.96±1.1 ab

Total motility (MT), progressive motility (MP), mean distance traveled (DAP), curvilinear distance (DCL), rectilinear distance (DSL), lateral head displacement (ALH), frequency of cross flagellar beating (BCF), dimethylsulfoxide (DMSO), glycerol (GLY) and methyl glycol (MG). Different letters indicate differences (P<0.05)

Continued Table - 1. Analyses of average path velocity, curvilinear velocity, straightness, linearity and oscillation of *Austrolebias minuano* (Costa & Cheffe, 2001), with dimethylsulfoxide, glycerol and methyl glycol alcohols at different concentrations (mean ± standard error)

	Spermatic Kinetics				
Treatments (%)	**VAP**	**VCL**	**STR**	**LIN**	**WOB**
DMSO 5	13.34±1.6^{def}	17.72±2.1^{cd}	0.29±0.0^{cde}	0.23±0.0^{cde}	0.29±0.0^{de}
DMSO 7.5	9.35±1.3^{fgh}	12.89±1.7^{def}	0.23±0.0^{ef}	0.17±0.0^{ef}	0.21±0.0^{fg}
DMSO 10	17.18±1.5^{bcd}	23.77±2.1^{b}	0.41±0.0^{ab}	0.30±0.0^{ab}	0.38±0.0^{abc}
DMSO 12.5	11.57±1.2^{efg}	16.66±1.7^{cde}	0.28±0.0^{de}	0.20±0.0^{de}	0.28±0.0^{def}
DMSO 15	13.86±1.3^{cde}	20.35±2.1^{bc}	0.32±0.0^{cd}	0.23±0.0^{cde}	0.31±0.0^{cd}
GLY 5	10.78±1.3^{efg}	15.10±1.8^{cdef}	0.27±0.0^{de}	0.20±0.0^{de}	0.25±0.0^{def}
GLY 7.5	16.89±2.5^{bcd}	23.83±2.99^{d}	0.37±0.0^{bc}	0.26±0.0^{bcd}	0.32±0.0^{bcd}
GLY 10	8.31±1.1^{gh}	11.26±1.4^{ef}	0.23±0.0^{ef}	0.18±0.0^{ef}	0.22±0.0^{efg}
GLY 12.5	8.45±1.1^{gh}	11.73±1.6^{ef}	0.22±0.0^{ef}	0.17±0.0^{ef}	0.22±0.0^{efg}
GLY 15	6.53±0.9^{h}	9.64±1.5^{f}	0.17±0.0^{f}	0.12±0.0^{f}	0.16±0.0^{g}
MG 5	9.86±1.2^{efgh}	13.12±1.7^{def}	0.27±0.0^{de}	0.21±0.0^{de}	0.25±0.0^{def}
MG 7.5	22.97±1.6^{a}	32.55±2.2^{a}	0.47±0.0^{a}	0.34±0.0^{a}	0.44±0.0^{a}
MG 10	20.79±1.6^{ab}	32.56±2.6^{a}	0.43±0.0^{ab}	0.29±0.0^{abc}	0.39±0.0^{ab}
MG 12.5	15.81±1.2^{cd}	23.50±1.9^{b}	0.42±0.0^{ab}	0.30±0.0^{ab}	0.39±0.0^{ab}
MG 15	17.86±1.3^{bc}	25.74±2.1^{b}	0.43±0.0^{ab}	0.33±0.0^{a}	0.43±0.0^{a}

Path velocity (VAP), curvilinear velocity (VCL), straightness (STR), linearity (LIN), oscillation (WOB), dimethylsulfoxide (DMSO), glycerol (GLY) and methyl glycol (MG). Different letters indicate differences (P<0.05)

Likewise, for flow cytometry parameters, 7.5% concentration of MG was efficient in relation to mitochondrial functionality (MIT), membrane functionality (MFU), cell integrity (CD), DNA integrity (DNA) and production of reactive oxygen species (ROS) (Table 2). Among the treatments, DMSO 12.5% was the least effective for most of the analyzed parameters (Table 2).

Table - 2. Analyses of mitochondrial functionality, membrane functionality, cellular disruption, fluidity membrane, DNA fragmentation index, concentration of reactive oxygen species and lipid peroxidation of *Austrolebias minuano* (Costa & Cheffe, 2001), with dimethylsulfoxide, glycerol and methyl glycol alcohols at different concentrations (mean ± standard error)

	Flow cytometry						
Treatments (%)	**MIT**	**MFU**	**CD**	**MFL**	**DNA**	**ROS**	**LPO**
DMSO 5	84.71±2.1^{a} bc	72.92±2.1^{d} ef	29.95±3.1^{a} bc	45.64±2.8^{bc}	4.62±0.1 bcd	4561.0±747.91^{def}	15.17±1.4 abc
DMSO 7.5	81.66±2.5^{c}	81.26±1.1^{a} bc	31.08±3.2^{a} b	48.10±3.9^{bc}	4.25±0.1 de	5121.8±569.33^{def}	15.74±1.0 ab
DMSO 10	87.93±1.1^{a} b	76.46±2.1^{b} cde	21.30±1.0^{f}	34.11±3.2^{d}	4.32±0.2 de	5351.3±971.40^{def}	13.70±1.1 abcd
DMSO 12.5	88.46±0.9^{a}	63.33±3.9^{g}	23.58±0.9^{d} ef	47.81±2.6^{bc}	4.98±0.1 ab	11813±3914.6^{ab}	15.43±1.4 ab
DMSO 15	86.13±0.9^{a} bc	69.26±3.4^{fg}	25.16±1.0^{c} def	59.94±3.0^{a}	4.66±0.1 bcd	6389.9±924.09^{def}	12.72±1.2 bcde
GLY 5	84.60±1.3^{a} bc	81.06±1.1^{a} bc	21.73±1.0^{e} f	42.00±2.7^{cd}	4.55±0.0 bcd	5299.6±916.60^{def}	11.68±1.2 cde
GLY 7.5	87.20±1.3^{a} bc	77.53±2.1^{a} bcd	23.45±1.2^{d} ef	50.48±3.1^{abc}	4.41±0.1 cde	11533±1643.9^{abc}	17.03±1.2 a
GLY 10	85.46±1.5^{a} bc	70.60±2.0^{ef}	26.19±2.1^{a} bcdef	49.72±3.8^{bc}	4.05±0.1 e	4832.3±455.99^{def}	13.52±0.9 abcd
GLY 12.5	85.28±1.6^{a} bc	80.50±3.2^{a} bc	28.16±2.4^{a} bcd	42.37±3.7^{cd}	5.42±0.1 a	2539.8±244.75^{ef}	9.41±1.2^{e}
GLY 15	82.40±1.7^{b} c	83.46±2.2^{a}	27.11±1.9^{a} bcde	48.11±3.5^{bc}	4.22±0.1 de	5252.7±1008.4^{def}	11.25±1.2 de
MG 5	83.80±3.1^{a} bc	80.00±2.9^{a} bc	31.30±2.9^{a}	52.33±3,9^{ab}	4.62±0.1 bcd	7557.7±1144.3^{bcde}	13.88±1.3 abcd
MG 7.5	84.06±2.2^{a} bc	79.46±1.1^{a} bcd	25.63±1.9^{b} cdef	46.35±3.5^{bc}	3.96±0.2 e	6694.5±1218.2^{cdef}	16.38±1.2 a
MG 10	85.07±2.4^{a} bc	79.21±2.3^{a} bcd	23.12±1.6^{d} ef	45.91±3.5^{bc}	4.39±0.2 cde	3414.0±308.84^{ef}	13.60±1.4 abcd
MG 12.5	82.26±3.2^{b} c	82.60±1.1^{a} b	22.64±1.3^{d} ef	45.02±3.5^{bc}	4.82±0.1 bc	9464.4±1605.1^{abcd}	16.89±1.3 a
MG 15	83.06±3.2^{a} bc	75.50±3.1^{c} def	26.09±1.8^{a} bcdef	44.44±3.5^{bc}	4.31±0.1 de	13812±4186.8^{a}	14.52±1.4 abcd

Mitochondrial functionality (MIT), membrane functionality (MFU), cellular disruption (CD), fluidity membrane (MFL), DNA fragmentation index (DNA), concentration of reactive oxygen species (ROS), lipid peroxidation (LPO), dimethylsulfoxide (DMSO), glycerol (GLY) and methyl glycol (MG). Different letters indicate differences (P<0.05)

4 DISCUSSION

This was the first study to test the protocol for sperm cryopreservation in annual fish, where the class of internal cryoprotectants (penetrants) was tested. The treatments evaluated with the use of methyl glycol proved to be efficient in almost all parameters related to sperm motility, a fundamental property for the reproductive success of the species (Cabrita et al., 2010; Maria et al., 2006). In fact, when compared to DMSO and GLY, the MG in its concentration of 7.5% obtained the most efficient results regarding progressive motility, presenting values up to three times higher than the other alcohols. In addition, 7.5% of the MG presented the best results of sperm kinetics, showing twice as good as the other cryoprotectants in relation to distance (DAP) and velocity (VAP). These results are congruent with those presented by Pereira et al. (2018) for Suruvi Steindachneridion scriptum, where MG was more effective.

However, the advantageous characteristics of MG were not restricted only to sperm motility parameters, since the effectiveness of this cryoprotectant can be further confirmed in many of the flow cytometric analyzes. In fact, the MG 7.5 treatment conferred low levels of DNA fragmentation (DNA), showing that DNA integrity was maintained during freezing and after thawing, and lower rates of lipid peroxidation. The number of reactive oxygen species (ROS) was shown to be reduced in almost all evaluated treatments, which is important for the other parameters, because the lower the number of ROS, the lower the chances of damage to the membrane, and consequently the better the membrane fluidity. According to Silva et al. (2016), this also improves mitochondrial functionality. In this sense, mitochondrial and membrane functionality, as well as rates of cell disruption and membrane fluidity, are also among the parameters for which several treatments performed similarly.

The effect of MG on fish was first tested in the cryopreservation of semen in Piracanjuba (Maria et al., 2006), where it was more effective. The methyl glycol cryoprotectant is derived from methanol (CH3OH) and ethylene oxide (CH2OCH2) (Viveiros, Orfão, Maria, & Allaman, 2009b), being considered a non-toxic alcohol (Takagi, Otoi, & Suzuki, 1993), which may result in more desirable discoveries when compared to other alcohols, such as DMSO (Viveiros, Oliveira, Maria, Orfão, & Souza, 2009a). In this sense, Viveiros et al., (2011) they compared DMSO and methyl glycol as cryoprotectants, at a concentration of 10%, for Brycon insignis and found better sperm motility results with the use of methyl glycol (77% to 82%) compared to DMSO (23% a 46%). In a study with Brycon nattereri, Viveiros et al. (2012) also show that cryopreserved semen with methyl glycol combined with BTS® has high quality. Oliveira et al., (2007), in one of his experiments using four different freezing media, observed the highest sperm motility (66%) in freezing in BTS® + methyl glycol. BTS® has 80% glucose in its composition and has been increasingly used in cryopreservation of fish semen. In piracanjuba, sperm motility was observed above 60% in frozen semen in BTS® + methyl glycol (Maria et al., 2006).

DMSO is the cryoprotectant most commonly used in freshwater fish in concentrations of 5 - 15% (Viveiros & Godinho, 2009), being considered as one of the best cryoprotectants due to its efflux capacity and cellular inflow, in a process that is much less temperature dependent than other cryoprotectants (Suzuki, Komada, Takai, Arii, & Kozima, 1995). In the present study, DMSO in its intermediate concentration of 10%, showed a better result in almost all the kinetic parameters, presenting a reduction of performance in smaller concentrations. However, the toxic effects of DMSO on fish, when used in high concentrations or when the exposure time is extended, is still little studied (Gwo, Jamieson, & Leung, 2009; Robles, Cabrita, Real, Álvarez, & Herráez, 2003). On the other hand, there are studies that consider, like ours, that DMSO is not the best cryoprotectant for fish semen freezing (Menezes et al., 2008).

Finally, glycerol presented the lowest values for all kinetic parameters evaluated in the establishment of a protocol for cryopreservation of *A. minuano* semen. According to the concentrations tested, 7.5% was the least toxic concentration, presenting the highest values within the cryoprotectant, while at its highest concentration (15%), the values showed to be more toxic. The negative results observed with the use of glycerol can be due to the fact that it can cause a toxic effect to the sperm cell, there being protein denaturation and modifications in the actin interactions of the spermatozoa tail, which can interfere in the spermatozoa movement and, consequently, in the process of fertilization (Alvarenga, Landim-Alvarenga, Moreira, & Cesarino, 2000). Similar results were obtained, for example, for equine semen (Pace & Sullivan, 1975) and in Indian red jungle (Blesbois et al., 2007; Rakha et al., 2016, 2017).

It is known that, in similar studies, percentages equal to or greater than the results observed here were obtained. However, for an endangered species, the spermatic freeze of *A. minuano* presents percentages of motility that are efficient for its level of threat; that is to say, although low, the mentioned percentages are significantly relevant when considered for a species that is threatened.

Based on these results, we propose that the use of cryoprotectants is effective for the spermatic preservation of the annual fish *Austrolebias minuano*. In addition, it is emphasized that there is a high sensitivity of these spermatozoa to glycerol and we recommend that their use should be avoided for preservation purposes. Likewise, the study shows that the combination of BTS® and methyl glycol 7.5% showed the best results, both for sperm kinetic parameters and integrity and cell biochemistry parameters.

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CONFLICT OF INTEREST

None.

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CONCLUSÕES E PERSPECTIVAS

Austrolebias minuano faz parte de populações únicas, que vivem em pequenas poças e que se encontram, frequentemente, isoladas (GIBBS, 2000). Essa característica, a construção de estradas, a agricultura e o crescimento urbano acelerado podem levar essa espécie à extinção. Além disso, barreiras de isolamento reprodutivo podem facilitar os processos de vicariância e especiação alopátrica em populações de peixes anuais (BEROIS; GARCÍA; DE SÁ, 2015; RITCHIE, 2007). Os trabalhos realizados nessa dissertação, em conjunto, permitiram a caracterização dos níveis de diversidade e estruturação genética entre populações já delimitadas de *Austrolebias minuano*, além de possibilitar a escolha das populações para realização de processos de preservação do material genético.

Nossas análises revelaram que, além de vários casos de níveis significativamente altos de diferenciação entre grupos de populações, *A. minuano* é subdividida em pelo menos três diferentes grupos populacionais, além de *Austrolebias pongondo*. Isso sugere que a vicariância desempenhou um papel importante na diversificação genética de *A. minuano*, corroborando com as nossas hipóteses. Além disso, a fragmentação alopátrica parece ser um processo contínuo dentro de *A. minuano*, onde os valores significativos e altos de FST encontrados entre os quatro grupos de populações de *A. minuano* e *A. pongondo* também evidenciam a ocorrência desses processos.

Foi verificado que a Lagoa dos Patos age como uma barreira entre o grupo de população minuano 1 e minuano 2 e 3. Além disso, a diferenciação encontrada entre todos os grupos, pelas análises concatenadas, induz que pelo menos alguns desses grupos podem se constituir de espécies incipientes. Dessa forma, estudos anteriormente realizados, utilizando técnicas moleculares, em espécies de peixes anuais mostraram a descoberta de novas espécies (COSTA, 2013). No entanto, argumenta-se aqui que é de extrema importância o emprego de análises morfológicas em cada uma das populações estudadas, para confirmar se esses grupos são novas espécies de peixes anuais.

Ademais, o processo de congelamento e a comparação entre os crioprotetores utilizados, mostraram que é possível realizar o processo de criopreservação de sêmen para o peixe anual *A. minuano* e que entre os álcoois e diferentes concentrações utilizadas é possível distinguir a toxicidade de algumas concentrações e crioprotetores. Portanto, sugere-se que o uso de métodos de criopreservação, como instrumento de preservação do patrimônio genético, seja adotado para cada uma das populações de *Austrolebias minuano*. Espera-se, também, que as informações adquiridas por esses estudos sejam utilizadas na implementação de programas que incentivem a proteção desses peixes anuais, e por consequência, desses ambientes.

Ainda que a presente dissertação tenha auxiliado na explicação de algumas questões relativas à evolução e utilização da criopreservação como técnica de armazenamento genético em espécies de *A. minuano*, é necessário que novos estudos evidenciem alguns aspectos importantes, tais como a realização de análises de diversidade para todas as espécies que estão inseridas no grupo *Austrolebias adloffi*, realizar avaliações morfológicas em todas as populações que foram utilizadas neste estudo, testar outros crioprotetores internos, comparar diferentes formas de resfriamento lento e vitrificação para o sêmen de *A. minuano*, bem como aprimorar o protocolo de criopreservação para outras espécies pertencentes à família Rivulidae.

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