UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE ANIMAL

Marcelo Schuler Crivellaro

FILOGEOGRAFIA DE *Aegla longirostri* (CRUSTACEA, DECAPODA, ANOMURA)

Santa Maria, RS, Brasil
2017

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Biodiversidade Animal, Área de Concentração em Sistemática e Biologia Evolutiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Ciências Biológicas – Área Biodiversidade Animal.**

Orientadora: Dra. Marlise Ladvocat Bartholomei-Santos

Co-orientadora: Dra. Bianca Laís Zimmermann

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2017

Ficha catalográfica elaborada através do Programa de Geração Automática
da Biblioteca Central da UFSM, com os dados fornecidos pelo(a) autor(a).

Schuler Crivellaro, Marcelo
Filogeografia de Aegla longirostri (Crustacea, Decapoda, Anomura) / Marcelo Schuler Crivellaro.- 2017.
64 p.; 30 cm

Orientadora: Marlise Ladvocat Bartholomei-Santos
Coorientadora: Bianca Laís Zimmermann
Dissertação (mestrado) - Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Programa de Pós-Graduação em Biodiversidade Animal, RS, 2017

1. Espécies Crípticas 2. Filogeografia 3. Sistemática Molecular I. Ladvocat Bartholomei-Santos, Marlise II. Zimmermann, Bianca Laís III. Título.

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Santa Maria, RS
2017

AGRADECIMENTOS

À UFSM e ao PPG Biodiversidade Animal por toda a estrutura.A todos os professores e colegas da Biologia por todo o conhecimento transmitido. À Capes pela bolsa concedida.

Aos meus pais, Luciane Duarte Schüler e Sergio Crivellaro, e aos meus irmãos, Nathalia e Rafael, por todo o apoio das diversas maneiras. Aos meus sobrinhos, Antonella, Helena e Vicenzo, que me matam de saudades e me enchem de alegria sempre que penso em vocês. Sem vocês nada faria sentido.

Para toda a família Crivellaro e Schüler que mesmo longe sempre estiveram comigo, e com certeza serviram de motivação e exemplo à minha formação. Em especial à tia Cerize, por todo o apoio em tudo e sempre, estará comigo eternamente!

À Laura Minussi Giuliani, por estar ao meu lado sempre, me apoiando e motivando nos momentos mais difíceis. Além de todo o amor, carinho e parceria durante todos esses anos e os que ainda virão.

Aos meus orientadores, Marlise Bartholomei-Santos e Sandro Santos, pela oportunidade que me deram de trabalhar com a Filogeografia, me fazendo enxergar a vida de outra maneira. Também pelo conhecimento transmitido, por terem acreditado no meu trabalho e por todas conversas sobre o passado dos eglídeos.

À minha co-orientadora, Bianca Zimmermann, por toda a paciência e sabedoria ao me ensinar todas as técnicas e análises que hoje sei. Não tem como medir o quanto tu contribuíste para a minha formação, obrigado!

Ao pessoal do laboratório (Aimée, Alberto, Alexandre, Amanda, Bianca, Ciço, Cris 1, Cris 2, Diogo, Fernando, Gláucia, Jô, Juliana e Marcelo) pelas conversas, risadas e momentos de descontração. Pela parceria nos 10 cafés diários e por proporcionarem um ambiente de trabalho tão legal. Não é sempre que se tem a oportunidade de sentar ao lado de doutores premiados e gringos oriundos de todos os cantos do Rio Grande e Paraná (queria ser gaúcho), quantos causos contados...Além disso, todas as discussões sobre artigos e métodos que com certeza contribuíram para minha formação.

Aos meus Brothers do Pipipitchú (George, Juliano e Renan) pela super parceria e por tornarem esses anos em SM mais engraçados.

RESUMO

FILOGEOGRAFIA DE *Aegla longirostri* (CRUSTACEA, DECAPODA, ANOMURA)

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Aegla é o gênero mais diversificado de caranguejos de água doce do sul da América do Sul. A família Aeglidae, a qual pertence este gênero, possui origem marinha, com registro de dois gêneros fósseis, encontrados em sedimentos marinhos na Nova Zelândia e México. Atualmente, são conhecidas 83 espécies de *Aegla* que ocorrem nas bacias hidrográficas do Brasil, Argentina, Bolívia, Chile, Paraguai e Uruguai, sendo encontradas desde 320 metros de profundidade até 3.500 metros de altitude. Aproximadamente 70% das espécies de *Aegla* encontram-se como ameaçadas de extinção, principalmente pela rápida degradação do ambiente aquático combinada com a distribuição restrita da maioria das espécies. Apesar do grande número de espécies descritas, seus caracteres morfológicos diagnósticos exibem pouca variação resultando em dificuldades na identificação correta das espécies. Além disso, a ocorrência de espécies crípticas em eglídeos já foi sugerida. Identificar espécies crípticas é essencial para estimativas mais precisas da biodiversidade, para entender os processos que levam à diversificação da vida e, também, para direcionamento correto de esforços de conservação, caso alguma delas estiver ameaçada. Evidências moleculares e de morfometria geométrica sugerem que a espécie nominal *Aegla longirostri* seja formada por um complexo de espécies, merecendo atenção especial em relação à sua real distribuição, endemicidade e status de conservação. Uma ferramenta que provou ser bem sucedida em revelar a biodiversidade críptica é a filogeografia. Utilizando diversas técnicas moleculares e métodos analíticos, a filogeografia testa hipóteses sobre a relação causal entre fenômenos geográficos, distribuições de espécies e os mecanismos que conduzem à especiação. A presente dissertação tem como principal objetivo estimar as relações filogenéticas e os padrões filogeográficos das populações de *Aegla longirostri*, e assim, testar a hipótese de que formam um complexo de espécies crípticas. Se for um complexo de espécies, identificar quantas e quais populações compõem as potenciais espécies crípticas. Além disso, objetiva-se analisar a estrutura genética das populações utilizando dois marcadores moleculares mitocondriais (COI e 16S) e um marcador molecular nuclear (íntron do gene ANT), afim de entender quais processos históricos podem ter influenciado na distribuição das linhagens genéticas. Foram analisadas 17 populações de *A. longirostri* e os resultados confirmaram a hipótese proposta, sendo que, métodos de delimitação de espécies indicaram que o complexo é formado por pelo menos 14 possíveis espécies. Além disso, os resultados observados sugerem uma possível contribuição da topografia da paisagem na diversificação desse complexo. Estudos futuros que busquem encontrar novos caracteres diagnósticos ou novas técnicas para a delimitação das espécies de *Aegla* são necessários. A real diversidade do gênero ainda é subestimada e é indispensável quantificar com precisão a sua diversidade oculta, e assim, aplicar medidas mais efetivas de gestão e conservação da biodiversidade.

Palavras-chave: Espécies Crípticas. Filogeografia. Sistemática Molecular.

ABSTRACT

PHYLOGEOGRAPHY OF *Aegla longirostri* (CRUSTACEA, DECAPODA, ANOMURA)

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Aegla is the most diverse genus of freshwater crabs in southern South America. The Aeglidae family, which belongs to this genus, has a marine origin, with two fossil genera, found in marine sediments in New Zealand and Mexico. Currently, 83 species of *Aegla* are known to occur in the watersheds of Brazil, Argentina, Bolivia, Chile, Paraguay and Uruguay, being found from 320 meters of depth to 3.500 meters of altitude. Approximately 70% of *Aegla* species are as endangered, mainly due to the rapid degradation of the aquatic environment combined with the restricted distribution of most species. Despite the high number of species described, their diagnostic morphological characters exhibit little variation resulting in difficulties in correctly identifying the species. In addition, the occurrence of cryptic species in eglids has already been suggested. Identifying cryptic species is essential for more accurate estimates of biodiversity, to understand the processes that lead to life diversification, and also for the correct direction of conservation efforts, if any are threatened. Molecular and geometric morphometric evidence suggest that the nominal species *Aegla longirostri* is formed by a complex of species, deserving attention in relation to its real distribution, endemicity and conservation status. One tool that has proven to be successful in revealing cryptic biodiversity is phylogeography. Using various molecular techniques and analytical methods, phylogeography tests hypotheses about the causal relationship between geographic phenomena, species distributions, and the mechanisms that lead to speciation. The present dissertation aims to estimate phylogenetic relationships and phylogeographic patterns of *Aegla longirostri* populations, and thus test a hypothesis that they form a complex of cryptic species. If it is a species complex, identify how many and which populations make up the potential cryptic species. In addition, we aimed to analyze the genetic structure of the populations using two mitochondrial molecular markers (COI and 16S) and a nuclear molecular marker (intron of the ANT gene), in order to understand which historical processes may have influenced the distribution of the genetic lineages. 17 populations of *A. longirostri* were analyzed and the results confirmed the hypothesis proposed, wherein, species delimitation methods indicated that the complex is formed by at least 14 possible species. In addition, the observed results suggest a possible contribution of the landscape topography in the diversification of this complex. Future studies that seek to find new diagnostic characters or new techniques for the delimitation of *Aegla* species are necessary. The real diversity of the genus is still underestimated and it is essential to accurately quantify its hidden diversity and, therefore, to apply more effective measures for the management and conservation of biodiversity.

Key-words: Cryptic Species. Phylogeography. Molecular Systematics.

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APRESENTAÇÃO

A presente dissertação, estruturada de acordo com as normas do Manual de Dissertações e Teses da UFSM (MDT), integra os requisitos necessários para a obtenção do título de Mestre em Ciências Biológicas – Área Biodiversidade Animal, pelo Programa de Pós-Graduação em Biodiversidade Animal da Universidade Federal de Santa Maria. Ela é composta por um texto integrador, um artigo e conclusões.

O texto integrador inclui inicialmente uma contextualização sobre o potencial dos estudos filogeográficos para desvendar espécies crípticas e a sua importância para a conservação da biodiversidade. Logo após, o organismo de estudo é apresentado, bem como os objetivos desta dissertação.

O artigo tem como foco as relações filogenéticas e os padrões filogeográficos das populações do complexo de espécies *Aegla longirostri*. O manuscrito é apresentado de acordo com as normas de formatação exigidas pelo periódico científico *Zoological Journal of the Linnean Society*, o qual o mesmo foi publicado.

Nas conclusões são apresentados os resultados obtidos ao longo deste estudo em relação aos objetivos apresentados ao final do texto integrado

1 – INTRODUÇÃO

1.1 – O gênero *Aegla*

Os crustáceos decápodos da superfamília Aegloidea Dana, 1852 constituem apenas um gênero atual, *Aegla* Leach 1820, com aproximadamente 83 espécies descritas (SANTOS et al., 2015; MORAES et al., 2016). O gênero atual é o único da infraordem Anomura que habita apenas águas continentais (BRACKEN-GRISSON et al., 2013). Eles ocorrem nas bacias hidrográficas do Brasil, Argentina, Bolívia, Chile, Paraguai e Uruguai, sendo encontrados desde 320 metros de profundidade em lagos chilenos, até 3.500 metros de altitude na pré-cordilheira Argentina. (BOND-BUCKUP et al., 2008). Os eglídeos são caranguejos pequenos (< 60mm), sendo normalmente encontrados debaixo de pedras, troncos submersos ou folhas depositadas sob o substrato de cursos d'água em rios, riachos, lagos e cavernas (BOND-BUCKUP et al., 2008).

A maioria das espécies de *Aegla* conhecidas apresenta distribuição restrita e são, portanto, de significativa preocupação quanto à conservação. Em um estudo recente que avaliou o status de conservação de 77 espécie de *Aegla,* 70% delas foram consideradas como ameaçadas de extinção (SANTOS et al., 2017), principalmente pelas suas distribuições restritas e a rápida degradação do ambiente aquático. Os eglídeos desempenham importante papel na reciclagem de nutrientes, participando do processo de fragmentação da biomassa de folhas que caem nos corpos d'água (COGO e SANTOS, 2013). Eles consomem detritos vegetais, algas, formas aquáticas imaturas ou adultas de insetos ou mesmo de outros crustáceos de pequeno porte e moluscos (MAGNI e PY-DANIEL, 1989; LARA e MORENO, 1995; BUENO e BOND-BUCKUP, 2004; SANTOS et al., 2008) e fazem parte da dieta de mamíferos, anfíbios, peixes, aves e répteis (ARENAS, 1974; MELO, 1990; CASSINI et al., 2009).

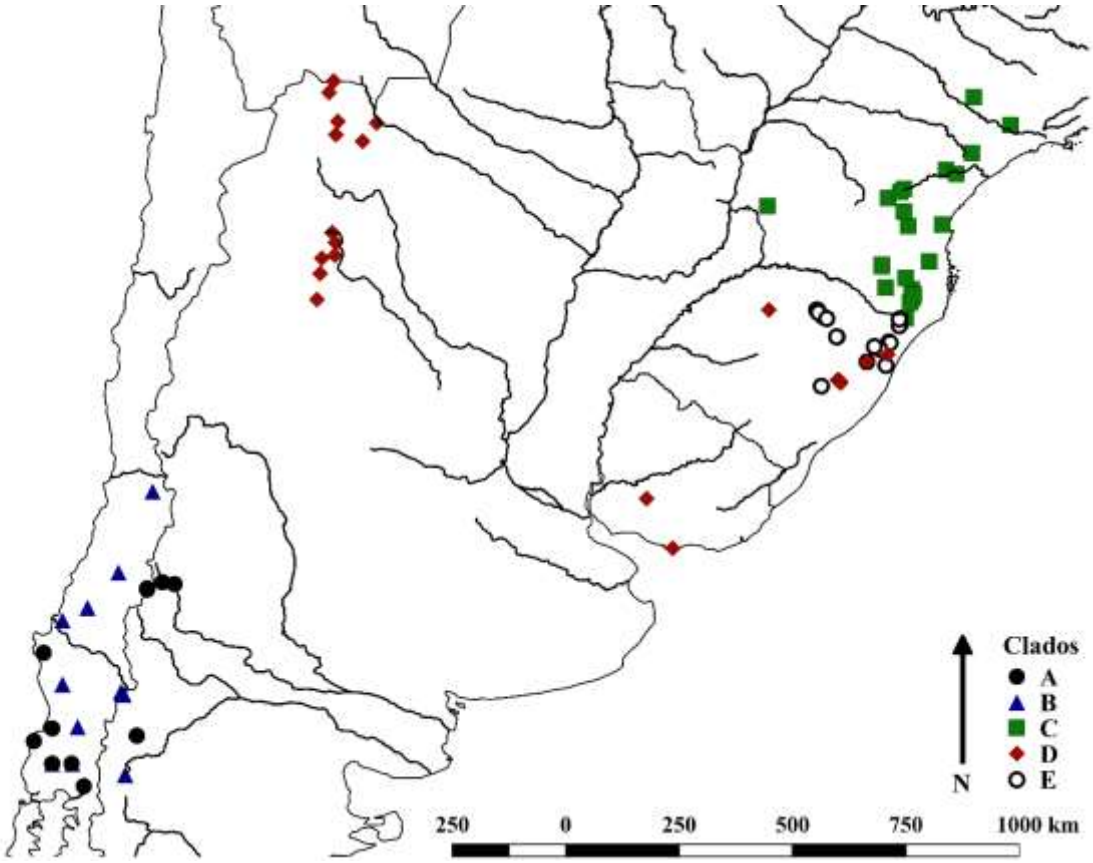
O grupo possui origem marinha, com registro de dois gêneros fósseis, *Haumuriaegla* e *Protaegla*, encontrados em sedimentos na Nova Zelândia (FELDMANN, 1984) e do México (FELDMANN et al., 1998), respectivamente. A rota utilizada pelos eglídeos para a transição do ambiente marinho para o ambiente de água doce já foi tema de muito debate. Ortmann (1902) propôs que as espécies Chilenas foram as primeiras a divergir, enquanto Schmitt (1942) e Ringuelet (1949) defendiam a hipótese de que os eglídeos do lado do Atlântico eram os mais ancestrais e que as espécies Chilenas eram mais derivadas. Com a descoberta do fóssil *Hamuriaegla,* a origem marinha dos eglídeos e sua irradiação pela América do Sul através da

costa do Chile passou a ser a hipótese mais aceita (FELDMANN et al., 1998). Ainda, Collins et al. (2011) sugerem que os ancestrais dos eglídeos poderiam ter se aproximado do México pela conexão entre o oceano Atlântico e o Mar do Caribe durante o meio e fim do Mioceno. Posteriormente, através das modificações provocadas na paisagem pelas transgressões marinhas, esses caranguejos podem ter dispersado ou, através de um evento vicariante, restringidos às zonas onde ocorrem atualmente.

Em um amplo estudo, Pérez-Losada et al. (2004) procuraram esclarecer aspectos da origem, posição taxonômica, biogeografia e sistemática de 64 espécies do gênero *Aegla* através de análises filogenéticas utilizando um gene nuclear (28S) e quatro genes mitocondriais (12S, 16S, COI e COII). Os dados encontrados indicaram que os eglídeos chilenos são as mais ancestrais e que a irradiação em águas continentais teria se iniciado há pelo menos 60 milhões de anos em decorrência de eventos de transgressões marinhas. A formação da Cordilheira dos Andes (iniciada há cerca de 90 milhões de anos) fez com que bacias hidrográficas que antes drenavam para o Pacífico passassem a drenar para o Atlântico (LUNDBERG et al., 1998), contribuindo para a dispersão dos caranguejos através do continente sul-americano.

Quanto a sistemática do grupo, os resultados de Pérez-Losada et al. (2004) reuniram as espécies em cinco clados filogenéticos (A-E): os clados A e B incluem as espécies chilenas e do sul da Argentina, enquanto que os clados C, D e E incluem as espécies do Norte da Argentina, Uruguai e Brasil (Figura 1). Os autores também observaram que seis espécies formaram grupos não-monofiléticos: *A. cholchol* Jara & Palacios, 1999, *A. franciscana* Buckup & Rossi, 1977, *A. jarai* Bond-Buckup & Buckup, 1994, *A. marginata* Bond-Buckup, 1994, *A. parana* Schimitt, 1942 e *A. platensis* Schimitt, 1942. Além dessas, *A. longirostri* Bond-Buckup 1994 demonstrou ser um grupo monofilético em análises de máxima verossimilhança e parafilético em filogenias que utilizaram máxima parcimônia. Baseado no conceito de que populações em que os grupos não são monofiléticos, provavelmente, representam diferentes espécies (CRACRAFT, 1983), as populações dentro desse taxa poderiam representar espécies distintas.

Figura 1. Mapa da distribuição geográfica dos cinco principais clados de eglídeos no Sul da América do Sul.



Fonte: Adaptado de PÉREZ-LOSADA et al. (2004).

1.2 - Filogeografia dos eglídeos

O termo filogeografia foi concebido há cerca de três décadas (AVISE et al., 1987) e a sua interpretação literal significa a análise filogenética de um organismo, no contexto de sua distribuição geográfica. Esses estudos utilizam diversas técnicas moleculares e métodos analíticos afim de testar hipóteses sobre a relação causal entre fenômenos geográficos, distribuições de espécies e os mecanismos que conduzem à especiação (AVISE, 2000). Desde que o termo surgiu, muitos avanços relacionados as técnicas moleculares, extensões da teoria da coalescência e outros métodos analíticos ampliaram o alcance da área dramaticamente. As perspectivas filogeográficas transformaram aspectos da biologia de populações, biogeografia, sistemática, ecologia, genética e conservação da biodiversidade (AVISE, 2016).

O DNA mitocondrial (mtDNA) tem sido o marcador mais utilizado para a reconstrução de padrões históricos da demografia de populações, biogeografia e especiação (HURST et al., 2005). Isso se deve ao fato de que o mtDNA acumula substituições de nucleotídeos a uma taxa várias vezes maior do que uma típica cópia de DNA nuclear, e essa rápida evolução é um pré-requisito para as análises filogeográficas (AVISE, 2009). O genoma mitocondrial dos animais é altamente compacto; isto é, não é sobrecarregado com os íntrons ou regiões intergênicas não codificadoras, que caracterizam os genomas nucleares. O mtDNA possui milhares de cópias por células e são representadas pela presença de 37 genes, todos ligados ao longo de uma molécula circular de aproximadamente 17.000 pares de nucleotídeos de comprimento. Além disso, o mtDNA é uma molécula "nua" (não é ligado às proteínas histonas, que são muito conservadas e podem restringir as taxas evolutivas no DNA nuclear) (AVISE, 2009). No entanto, interpretações evolutivas baseadas na utilização isolada do mtDNA são preocupantes. Autores sugerem que inferências sobre a estrutura populacional e delimitação de espécies devem ser corroboradas por outras linhas de evidência, geralmente na forma de dados oriundos de marcadores nucleares (ZINK et al., 2008). O mtDNA é transmitido de forma uniparental, com origem exclusivamente materna, ou seja, recupera apenas uma parte da história evolutiva e não sofre recombinação, sendo sua variação genética refletida apenas por mutações acumuladas ao longo do tempo (AVISE, 2009). Quando um táxon irmão diverge do ancestral comum a frequência de alelos de um determinado gene será inicialmente similar. Com o passar do tempo, a deriva genética irá alterar a frequência de alelos e novos alelos serão introduzidos em cada população através da mutação. Eventualmente as populações irão divergir e se diferenciar. Se analisarmos esses processos nos estágios iniciais, haverão alelos compartilhados entre as duas populações e elas aparecerão como polifiléticas ou parafiléticas em uma análise filogenética. Alelos idênticos podem ser encontrados em populações geneticamente distintas se o tempo de divergência do táxon irmão a partir de um ancestral comum não foi suficiente para permitir a fixação de alelos produzidos por mutação (AVISE, 2000). Por esses motivos que é de grande importância utilizar mais de um tipo de marcador, com taxas de substituições diferenciadas, a fim de evitar interpretações evolutivas de forma errônea.

Em um estudo utilizando três genes mitocondriais e três nucleares, Barber et al. (2012) demonstraram a importância de usar ambos os marcadores para um entendimento mais completo das histórias evolutivas e aos eventos filogeográficos associados. Os autores analisaram os padrões filogeográficos de *Aegla neuquensis*, espécie amplamente distribuída que ocorre em dois grandes sistemas fluviais, nas cabeceiras do sopé da Cordilheira dos Andes até o Oceano Atlântico. Devido ao fenômeno de introgressão mitocondrial, os padrões

filogeográficos encontrados para os diferentes marcadores não se repetiram, impossibilitando conclusões mais precisas.

Um dos eventos históricos mais bem estudados e conhecidos, que auxiliam nas explicações de padrões filogeográficos, são os episódios glaciais do Quaternário (2.6 milhões de anos atrás até o presente). Esses eventos foram um fator importante na modelagem das distribuições atuais da flora e fauna existentes, com expansões e contrações das manchas de gelo, tornando inabitáveis grandes áreas para a maioria das espécies. Além disso, as geleiras podem ter mudado os padrões de drenagem, as distribuições dos lagos e até a posição da divisão continental, resultando no deslocamento de populações de plantas e animais (CLAPPERTON, 1993). Os registros fósseis, corroborados pelos estudos filogeográficos, sugerem que muitas espécies apenas sobreviveram aos máximos glaciais ao ficarem restritas em refúgios (também conhecidos como refúgios pleistocênicos), locais que permaneceram habitáveis e que podem ter servido de fonte na colonização pós-glacial (PROVAN et al., 2008). Refúgios ou populações fontes podem ser rastreados ao comparar os seus perfis genéticos ao de populações de áreas colonizadas.

Xu et al. (2009) examinaram os impactos das glaciações do Pleistoceno nos padrões filogeográficos da espécie *Aegla alacalufi* Jara & López, 1981 na Patagônia Chilena. A espécie habita áreas continentais que foram cobertas por gelo, e também ilhas que não sofreram glaciação, sendo consideradas potenciais áreas de refúgio. Os resultados indicaram que as ilhas não serviram de refúgio às populações continentais. Por outro lado, as populações das ilhas são geneticamente mais estruturadas quando comparada com as populações continentais, indicando uma influência significante da glaciação nessas populações. Foram detectadas expansões demográficas no grupo continental, com um aumento populacional constante após o último máximo glacial, sugerindo que sua colonização pós-glacial teve como fonte algum outro refúgio.

1.3 - Espécies crípticas em *Aegla* e o caso *A. longirostri*

A filogeografia também provou ser muito bem sucedida em revelar a biodiversidade críptica, em alguns casos contestando a taxonomia tradicional dos grupos (AVISE, 2000; RIDDLE e HAFNER, 2006). Espécies crípticas são duas ou mais espécies diferentes, mas superficialmente indistinguíveis em termos morfológicos, que são erroneamente (e ocultamente) classificadas como uma só espécie (BICKFORD et al., 2007). Assim, identificar espécies crípticas é essencial não só para estimativas mais precisas, mas para entender os

processos que levam a diversificação da vida e para a conservação da biodiversidade (COOK et al., 2008), visto que a aglomeração incorreta de espécies distintas prejudica a conservação, caso alguma das delas estiver ameaçada (FRANKHAM et al., 2004).

Crustáceos decápodos tendem a abrigar um grau elevado de biodiversidade críptica (DA SILVA et al., 2011). Diversos estudos evidenciaram a presença de prováveis espécies crípticas em decápodos, como, por exemplo, nos gêneros *Potamonautes* (DANIELS et al., 2003), *Munida* (MARCHODOM et al., 2004), *Chaceon* (MANTELLATO et al., 2013) e *Macrobrachium* (CARVALHO et al., 2013). Espécies crípticas também já foram registradas em eglídeos (JARA et al., 2003; MORAES et al., 2016). É importante destacar que apesar do grande número de espécies descritas de *Aegla*, seus caracteres diagnósticos são limitados e a sua morfologia geral tem pouca variação (BOND-BUCKUP e BUCKUP, 1994). A morfologia relativamente conservada do gênero deixa poucos caracteres derivados compartilhados para resolver as relações evolutivas entre espécies (HEPP et al., 2012).

Dentre os eglídeos que poderiam compor agrupamentos crípticos, representando assim complexos de espécies, podemos destacar *A. longirostri*. Os motivos para tal premissa são expostos abaixo.

A maioria das espécies de *Aegla* possui distribuição restrita, aquelas com ampla distribuição, como *A. longirostri*, podem representar um complexo de espécies, merecendo atenção especial em relação à sua real distribuição, endemicidade e status de conservação (BARTHOLOMEI-SANTOS et al., 2011). A espécie ocorre no centro, leste e nordeste do estado do Rio Grande do Sul, sendo que, a região nordeste é caracterizada por sua superfície ondulada, o que pode contribuir para o isolamento de populações. Além disso, entre o fim do Pleistoceno e o início do Holoceno (~40,000 – 8,000 anos atrás) o clima nessa região era predominantemente seco (SPALDING et al., 2015) e pode ter isolado as populações de eglídeos em diferentes trechos dos rios, o que deve ter reduzido ou até impedido o fluxo gênico, o primeiro passo para especiação.

Bartholomei-Santos et al. (2011) investigaram a estrutura genética de quatro populações de *A. longirostri* no estado do Rio Grande do Sul, Brasil através de marcadores microssatélites. Uma dessas populações estava localizada em uma bacia hidrográfica diferente, estando geograficamente isolada das outras. Altos níveis de diferenciação genética foram encontrados entre as populações analisadas, e a maioria mostrou valores muito baixos de fluxo gênico, especialmente na população isolada. Essas diferenças foram encontradas mesmo na ausência de caracteres taxonômicos diagnósticos divergentes, o que reforça a hipótese que o grupo seria formado por espécies crípticas.

Em outro estudo, Marchiori et al. (2013) utilizaram como ferramenta a morfometria geométrica para analisar a forma da carapaça de seis populações de *A. longirostri*. Os dados morfométricos foram congruentes com os dados genéticos, de modo que as populações geneticamente diferenciadas também possuem diferenças na morfologia. Esses resultados corroboram a ideia de que a distância geográfica e o isolamento devem estar afetando o fluxo gênico entre as populações. O estudo sugeriu que *A. longirostri* pode ser formada por um complexo de espécies, ou que o grupo está em processo incompleto de especiação.

Considerando os altos níveis de divergência genética, as significativas diferenças morfométricas encontradas nas populações (BARTHOLOMEI-SANTOS et al., 2011; MARCHIORI et al., 2013) e a distribuição relativamente ampla em relação às outras espécies do gênero, *A. longirostri* é um organismo muito interessante para ser utilizado em estudos filogeográficos. Além disso, conhecer melhor o endemismo e a riqueza de espécies é fundamental para a preservação das mesmas sobretudo considerando que a biodiversidade aquática continental é uma das mais ameaçadas e necessita urgentemente de cuidados quanto à sua conservação (ABELL, 2002; PÉREZ-LOSADA et al., 2009).

2 - OBJETIVO GERAL

- Investigar os padrões filogeográficos e as relações filogenéticas das populações de *A. longirostri* a fim de testar a hipótese de que elas formam um complexo de espécies.

2.1 - OBJETIVOS ESPECÍFICOS

- Analisar a estrutura genética das populações de *Aegla longirostri* utilizando dois marcadores moleculares mitocondriais (COI e 16S) e um marcador molecular nuclear (íntron do gene ANT – *Adenine Nucleotide Transporter*);

- Entender quais processos históricos podem ter influenciado a distribuição das linhagens genéticas;

- Se for um complexo de espécies, identificar quantas e quais populações compõem as potenciais espécies crípticas.

3 - ARTIGO

Comprovante de publicação do artigo

Zoological Journal of the Linnean Society, 2017, **XX**, 1–14. With 3 figures.

Looks can be deceiving: species delimitation reveals hidden diversity in the freshwater crab *Aegla longirostri* (Decapoda: Anomura)

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Received 4 November 2016; revised 19 March 2017; accepted for publication 1 May 2017

Aegla is the most abundant and diversified genus of freshwater crabs from southern South America. Despite the high number of species described, their morphological diagnostic characters are not fully informative and exhibit little variation, which could result in cryptic species, as subtle interspecific differences can be easily overlooked. The aim of our study was to estimate the phylogenetic relationships and phylogeographic patterns of diversity of *Aegla longirostri* to test the hypothesis that this species represents a complex of cryptic species. We analysed 17 populations using three molecular markers. The analysis showed that *A. longirostri* constitutes a polyphyletic group; our trees suggest the presence of two major clades composed of many well-supported subclades that, despite being geographically close, are genetically distinct from each other. Surprisingly, species delimitation methods indicated the presence of at least 14 potential species. Our results suggest that the real diversity of aeglids may be largely underestimated and we discuss the conservation implications of cryptic diversity for this group. Given that prioritization of habitats for conservation often relies on estimation of species richness, endemism and conservation status coupled with the fact that several *Aegla* species are endangered, it is imperative to accurately quantify the hidden diversity of aeglids.

ADDITIONAL KEYWORDS: biodiversity – cryptic species – molecular systematics – phylogeography – South America.

Looks can be deceiving: species delimitation shows hidden diversity within freshwater crab, *Aegla longirostri* (Decapoda, Anomura)

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Running title: Cryptic diversity within freshwater crab

ABSTRACT

Aegla is the most abundant and diversified genus of freshwater crabs from southern South America. Despite the high number of species described, their morphological diagnostic characters are limited and exhibit little variation. This limited morphological variation could result in cryptic species, since subtle interspecific differences can easily be overlooked. The aim of our study was to estimate the phylogenetic relationships and phylogeographic patterns of diversity of *Aegla longirostri* to test the hypothesis that this species represents a complex of cryptic species. We analyzed 17 populations using two mitochondrial (16S rRNA and COI) and one nuclear (ANT) molecular markers. The analysis showed that *A. longirostri* constitutes a polyphyletic group; our trees suggest the presence of two major clades composed of many well-supported subclades that, despite being geographically close, are genetically distinct from each other. Surprisingly, species delimitation methods indicated the presence of at least 14 putative species. We also highlight a possible contribution of landscape topography to crab diversification. Our results suggest that the real diversity of aeglids may be largely underestimated and we discuss the conservation implications of cryptic diversity for this group. Given that prioritization of habitats for conservation often relies on estimation of species richness, endemism, and conservation status coupled with the fact that several *Aegla* species are endangered, it is imperative to more accurately quantify the hidden diversity of aeglids.

ADDITIONAL KEYWORDS: biodiversity - Brazil - cryptic species complex - molecular systematics – phylogeography.

INTRODUCTION

Species are often the central currency of biodiversity studies and associated conservation actions (e.g. Orme *et al.,* 2005; Agapow *et al.,* 2004). Therefore, delineating species boundaries correctly is crucial to the discovery and accurate cataloging of biodiversity (Choi, 2016). Species delimitation is traditionally assessed by comparing variation in morphological traits and, in some cases, physiological, developmental, behavioral, and/or ecological traits. Increasingly, however, genetic markers have been effectively used to delineate species boundaries (Yang & Rannala, 2010, 2014). For practical and historical reasons, most species have been primarily described based on morphology (Dayrat, 2005). Nonetheless, there are some disadvantages to relying solely on morphological information: there is always a subjective component when defining and interpreting character states; the continuous rather than discrete nature of many characters on which taxonomists heavily rely; and their unsuitability for some groups of organisms, because speciation can occur without morphological change, which leads to morphologically cryptic species (see Padial *et al.*, 2010 for review). By definition, cryptic species represent superficially indistinguishable morphologies, but in fact constitute taxa that are genetically divergent and reproductively isolated from each other. These species are either differentiated by nonvisual mating signals and/or appear to be under selection that promotes morphological stasis (Bickford *et al.,* 2007). Cryptic species, thus, represent units of evolution that are important for conservation, rather than conglomerations of morphologically similar species, which may not even be closely related (Page & Hughes, 2007).

In recent decades, molecular tools have gained great popularity among systematic biologists, in particular those studying decapods (Martin, Crandall & Felder, 2009). Indeed, molecular characters (e.g., DNA sequences) have been dramatically more successful than

morphology to resolve taxonomic conflicts and to detect cryptic and/or polymorphic species (Xiao *et al.*, 2010; Puillandre *et al.*, 2011; Bracken-Grisson *et al.*, 2012). The occurrence of cryptic species has been reported in all phyla and seems to be a frequent trend associated with morphological taxonomy (Lefébure *et al.*, 2006). In this sense, phylogeographic information provides an objective tool to aid in investigations of species delimitation and to help define boundaries for cryptic species, especially for organisms displaying fine-scaled endemism (Rissler & Apodaca, 2007). High quality data derived from phylogeographic sampling strategies (i.e., geographically dense and large sample sizes, molecular genetic-based phylogenies) can potentially resolve a single taxonomic species into two or more geographically distinct cryptic evolutionary lineages, frequently indicating that the original taxon is paraphyletic or polyphyletic (Riddle & Hafner, 2006). Moreover, knowledge of intraspecific molecular genealogies allows us to infer how paleoclimatic processes affected the dynamics of populations and determined the current genetic structure (Avise, 2009; Hickerson *et al.*, 2010). Therefore, using molecular information, particularly DNA sequences, seems to be of utmost importance to the understanding the role of cryptic species in shaping our global diversity patterns.

Aegla Leach, 1820 is the most abundant and diversified genus of freshwater crabs from southern South America, occurring in hydrographic basins in Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay. This genus is the only extant representative of the family Aeglidae, the sole anomuran family entirely restricted to freshwaters (Bracken-Grisson *et al.*, 2013). The invasion of continental waters by marine ancestors and subsequent diversification in this environment began at least 60 Mya (Pérez-Losada *et al.*, 2004). Currently, there are almost 80 species described (Santos *et al.*, 2013, 2015), which are grouped in five major phylogenetic clades based on molecular characters (Pérez-Losada *et al.*, 2004). Despite the high number of *Aegla* species described, their diagnostic characters are limited and their general morphology

has little variation among species (Bond-Buckup & Buckup, 1994). The relatively conserved morphology of the genus leaves few shared derived characters for resolving evolutionary relationships among species (Hepp *et al.*, 2012), which is highlighted by the fact that morphologically cryptic species have already been reported in aeglids (Jara, Pérez-Losada & Crandall, 2003). Additionally, in a large study that evaluated the molecular phylogeny of the *Aegla* species, six of these species were considered paraphyletic (Pérez-Losada *et al.*, 2004), reinforcing the idea that morphological and genetic diversity are not necessarily well aligned (Bisby, Coddington & Thrope, 1995).

Aegla longirostri Bond-Buckup and Buckup, 1994 is one of those particular cases. It has a relatively wide distribution when compared to other species (northeastern, central and eastern regions of Rio Grande do Sul state, Brazil), and it occurs in different river basins. Furthermore, when investigated using microsatellite markers, four populations of *A. longirostri* showed high levels of genetic differentiation and low gene flow (Bartholomei-Santos, Roratto & Santos, 2011). When considering morphology, geometric morphometric analysis of carapace shape also revealed considerable variation among six *A. longirostri* populations (Marchiori, Bartholomei-Santos & Santos, 2014). In both cases, differences were found even in the absence of divergent taxonomic diagnostic characters. Therefore, based on these three lines of evidence (i.e., distribution, molecular characters, and morphology), we hypothesize that *A. longirostri* represents a complex of cryptic species that requires further attention. Thus, the aim of this study was to use three DNA molecular markers (two mitochondrial and one nuclear) and phylogenetic and phylogeographic approaches to investigate the evolutionary history and cryptic diversity of the nominal ‘species’ *A. longirostri*.

MATERIAL AND METHODS

SAMPLE COLLECTION

A total of 186 specimens were sampled from 17 locations across Jacuí River basin in the Rio Grande do Sul state, covering the major geographical range of the species (Fig. 1; and Table 1) and including the type-locality at Rolante municipality. Individuals were preserved in ethanol and identified on the basis of their external morphology (Bond-Buckup & Buckup 1994). Tissue (gill or muscle) samples were used for molecular procedures. Genetic vouchers, from which tissue samples were obtained, were deposited at the Crustacean Collection of the Department of Ecology and Evolution, Federal University of Santa Maria (UFSM), Brazil. For outgroups, we included *A. abtao* Schmitt, 1942, *A. riolimayana* Schmitt, 1942 and *A. spinipalma* Bond-Buckup and Buckup, 1994.

DNA EXTRACTION, PCR AMPLIFICATION, AND SEQUENCING

Genomic DNA was extracted using QIAamp DNA Mini Kit (QIAGEN) procedures following the manufacturer's instructions. Partial fragments of two mitochondrial genes, 16S ribosomal RNA (16S) and cytochrome c oxidase subunit I (COI) were amplified with primers 16Saeglid-f/16Saeglid-r (Pérez-Losada *et al.*, 2002) and LCOI-f/COIA2-r (Xu *et al.*, 2009). We also amplified a nuclear intron fragment of the adenine nucleotide transporter gene (ANT) using the primer pair DecapANTF/ANTir1 (Teske & Beheregaray, 2009; Barber *et al.*, 2012). Standard polymerase chain reaction (PCR) was run and PCR products were checked by agarose gel electrophoresis, purified and then sequenced. Sequences were aligned with Muscle (Edgar, 2004). All contiguous insertion/deletion events (indels) were treated as one mutational step (Simmons, Ochoterena & Carr, 2001) and hypervariable sites were weighted as zero to prevent the inclusion of homoplastic characters.

PHYLOGENETIC ANALYSES

Phylogenetic analyses were conducted for mitochondrial, nuclear, and concatenated (mitochondrial + nuclear) data sets using maximum-likelihood (ML) and Bayesian methods with gene partitions. The best-fit models of nucleotide substitution for each gene were selected with JModeltest 2.1.10 (Darriba *et al.,* 2012). ML analyses were performed with raxmlGUI 1.5 (Silvestro & Michalak, 2012), using a GTR+CAT model with nodal support estimated by 1000 bootstrap replicates. Bayesian analyses were performed using the Monte Carlo Markov Chain (MCMC) method as implemented in BEAST version 1.8.0 (Drummond *et al.,* 2012). The Bayesian analysis was run for 50 million chains and sampled every 1,000 generations. Posterior probabilities were calculated with a burn-in of 5 million states and checked for convergence using Tracer version 1.6 (Rambaut *et al.,* 2014). To test the monophyly and placement of the sequences of *A. longirostri* according the clades defined by Pérez-Losada *et al.,* (2004), a Bayesian phylogeny was conducted in the BEAST program using mitochondrial sequences (COI and 16S) of individuals from this study and from other species of *Aegla* deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/), including representatives of the major clades described. The analysis was run for 30 million generations and sampled every 1,000 generations. Posterior probabilities were calculated with a burn-in of 3 million generations. All results were visualized and checked with FigTree 1.4.2 (Rambaut 2014).

SPECIES DELIMITATION

We tested for the threshold between interspecific and intraspecific diversification using two species delimitation analyses with mitochondrial gene sequences (COI + 16S): (i) the General Mixed Yule Coalescent (GMYC) method of Pons *et al.,* (2006), and (ii) the Automatic Barcode Gap Discovery (ABGD) method of Puillandre *et al.,* (2012). Unlike ABGD that uses detection of the 'barcode gap' in the distribution of genetic pairwise distances, GMYC uses a phylogenetic input tree from which the fit of speciation and coalescent processes are modeled

to delineate molecular operational taxonomic units (MOTUs) (Flot, 2015). Additionally, GMYC is remarkably stable under a wide array of circumstances, including most phylogenetic reconstruction methods, high singleton presence, taxon richness and the presence of gaps in intraspecific sampling coverage (Talavera, Dincă & Vila, 2013). The ABGD method was implemented using the online version of the program (http://wwwabi.snv.jussieu.fr/public/abgd/) with default parameter and *P*min = 0.005/*P*max = 0.1. The GMYC method was implemented using an ultrametric tree estimated in BEAST. Species delimitation through the GMYC model was conducted using the standard parameters (interval = c(0, 10)) and a single threshold that specifies the transition time between to within species branching. These analyses were conducted with the package 'splits' (Species Limits by Threshold Statistics; http://r-forge.r-project.org/projects/splits) using R v.3.3 (R Development Core Team 2011).

Additionally, we attempted to delimit species by comparing species model hypotheses using a Bayes Factor (BF) approach for model selection. The tested models were: unconstrained tree (Model 1), constrained tree considering *A. longirostri* as monophyletic (Model 2), constrained tree considering species defined by ABGD (Model 3), and constrained tree considering species defined by GMYC (Model 4). We also tested the monophyly of the major clades found in this study (Model 5). BF calculates the ratio of the marginal likelihood of two models, which has the advantage of taking into account priors used in the Bayesian analysis (Xie *et al.,* 2011). It is worth mentioning that standard BF test is always biased toward acceptance of the monophyly hypothesis. Thus, one can safely use a standard BF test to reject a hypothesis of monophyly, but it is typically going to be an extremely conservative test (Bergsten, Nilsson & Ronquist, 2013). The marginal likelihood values of these competing models were estimated using stepping-stone sampling (SS, Xie *et al.*, 2011) in the BEAST package and run for ten million generations of 50 path-steps. The better model was chosen

when twice the natural logarithm of the Bayes-factor testing statistic (2*ln*Bf) was greater than 2 (Kass & Raftery, 1995). A value greater than 10 was assumed to indicate decisive support for distinguishing between competing species-delimitation hypotheses (Grummer, Bryson & Reeder, 2014). All parameters were setup as described in the previous section. This allows for the direct comparison of the two models considering both the topology and the branch lengths of species trees.

POPULATION GENETIC, PHYLOGEOGRAPHIC, AND DEMOGRAPHIC ANALYSIS

The levels of mtDNA haplotype diversity (h) and nucleotide diversity (π) were estimated for the inferred groups (populations) using DnaSP v. 4.10.3 (Rozas *et al.*, 2003). To examine the population structure, an analysis of molecular variance (AMOVA) was performed; its significance was tested by running 10,000 permutations in ARLEQUIN v. 3.1 (Excoffier, Laval & Schneider, 2005). The AMOVA was run with no hierarchical structure (all populations in a single group) and with pairwise comparisons between sampling sites. Genetic differentiation (F_{ST}) was computed using the same program. Additionally, mtDNA genetic divergences amongst and within populations were obtained using p-distance model with 1,000 bootstrap replicates in MEGA 6.0 (Tamura *et al.*, 2013).

Tajima's *D* (Tajima, 1989), Fu's *Fs* (Fu, 1997) and Fu and Li's *D** (Fu & Li, 1993) were applied to test the selective neutrality of genetic markers. These estimators are also sensitive to demographic processes such as recent population expansion or bottlenecks. Of these estimators Tajima's *D* and Fu's *Fs* are sensitive to an excess of recent mutations whereas Fu and Li's *D** can detect an excess of old mutations in a population that has experienced a historical reduction in effective population size. For each population, Tajima's *D* and Fu's *Fs* were estimated in ARLEQUIN with 10,000 simulations, and Fu and Li's *D** test was performed in DnaSP with 1,000 coalescent replicates.

SAMOVA v. 2.0 (Dupanloup *et al.,* 2002) was used to identify the geographic groupings that maximized genetic variance between groups of populations. The method calculates F statistics and identifies the optimum number of population groups for a set of sampled sites taking into account their geographic location. We used 100 simulated annealing processes for each value of K. The SAMOVA was run from K = 2 to the value of K that maximizes the value of the F_{CT} statistic.

The genealogical relationships among mitochondrial sequences were determined by a haplotype network generated with the median-joining method (Bandelt, Forster & Röhl, 1999) in NETWORK v. 4.6 (http://www.fluxus-engineering.com). Divergence times [i.e. time to the most recent common ancestor (TMRCA)] among haplotypes were estimated using a Bayesian approach with BEAST 1.8.0 (Drummond *et al.,* 2012). A run of 50 million chains were performed and sampled every 1,000 generations. The settings used were the Yule tree prior, the HKY+G+I substitution model with four gamma categories, and the strict molecular clock. The mtDNA substitution rate employed was 0.118 substitutions/site/million years (Xu *et al.,* 2009; Barber *et al.,* 2012), with a standard deviation of 5%.

RESULTS

PHYLOGENETIC ANALYSES

From the 186 individuals sampled, we obtained COI sequences for 186 specimens (723 bp unambiguous alignment), 16S rDNA for 179 specimens (412 bp aligned, including gaps), and nuclear ANT for 184 specimens (273 bp aligned, including gaps). The number of parsimony informative sites was 102 for COI, 43 for 16S and 16 for ANT sequences. Sequences were deposited in GenBank under the accession numbers KX910101 - KX910666.

Phylogenetic trees generated with single genes and with mitochondrial (Fig. 2) and concatenated sequences (Fig. 3) showed similar topologies, all highlighting the polyphyly of *A.*

longirostri. Additionally, two very distinct clades and several well-supported subclades were recovered. The pattern from the nuclear DNA was consistent with that from mtDNA, but with less resolution due to the low variation of this fragment (Fig. S1). Tree topologies were congruent between maximum likelihood and Bayesian analyses. Hence, only trees built with the latter method are presented.

Surprisingly, populations located in the Northern section of the distributional area (Northern clade) were genetically more related to the outgroup than to the populations from the Southern section (Southern clade), despite the geographical proximity between them. Moreover, when compared with sequences from other species of *Aegla*, it is evident that sequences from the Northern and Southern clade are very distinct from each other - according to the previous phylogenetic classification of Pérez-Losada *et al.*, (2004) (Fig. S2), they belong to clades E and D, respectively.

SPECIES DELIMITATION

The likelihood of the null model in the GMYC analysis (i.e., that all sequences belong to a single species) was significantly different from the maximum likelihood species delimitation (-544.21 *versus* -533.23, ratio = 21.97, $P = 1.7 \times 10^{-5}$). The GMYC analyses based on mtDNA datasets indicated the presence of 19 ML entities (including outgroups). Meanwhile, ABGD analysis with JC69 and K2P produced two recursive partitions with MOTUs counts of 17 ($P = 0.005$ and $P = 0.0069$) (Fig. 2). Both ABGD and GMYC methods indicated the presence of 10 putative species for the Northern clade, while GMYC indicated the presence of two additional species in the Southern clade when compared to ABGD (i.e., six *versus* four putative species).

Bayes Factor model selection results show that SS marginal likelihood estimators prefer Model 1 over Model 2 ($2lnBF = 7.12$), suggesting the paraphyly of *A. longirostri*. Also, BF

analysis strongly supports Model 3 and Model 4 over Model 1 ($2lnBF$ = 268.58 and 294.66, respectively), arguing in favor of the presence of multiple cryptic species. Indeed, models that considered the species delimitation defined by GMYC and ABGD were preferred over that which considered the monophyly of the two major clades recovered in this study (Model 5) (Table S1).

POPULATION GENETIC, PHYLOGEOGRAPHIC, AND DEMOGRAPHIC ANALYSIS

The genetic diversity estimates for the populations are summarized in Table 1. Forty-nine mitochondrial haplotypes (gaps were considered as the fifth state) were observed for the analyzed populations and all of them were population private. Haplotype diversity (h = 0.962) and nucleotide diversity (π = 0.038) were high when considering the entire data set, although these values were generally low when considering populations separately. The pairwise population differentiation estimates (F_{ST}) were also very high (F_{ST} ranging from 0.395 to 1.0) and highly significant for the majority of population comparisons (Table S2). Most of the genetic diversity was the result of variability among rather than within populations (AMOVA: among populations = 91.9%, within populations = 8.10%, *Fst* = 0.919, *P* = 0). The mean genetic divergence among populations was 3.4%, ranging from 0.1% (between Nova Palma and Rolante) to 5.08% (between Soledade and Caçapava do Sul). The mean genetic divergence within populations was 0.23% (Table S3).

For most populations, Tajima´s D showed no significant departure from zero (P > 0.05), as expected under neutral sequence evolution. The only exception was São Sepé (D = -1.629, P = 0.034). The F_S values were negative and statistically significant for Vale do Sol, Ivorá and São Sepé, supporting population expansion in these cases. For the other populations, no significant departures from equilibrium values were observed. Fu and Li's D* test was significant only for São João do Polêsine, indicating a recent bottleneck of population size

and/or balancing selection (Fu and Li's $D^* = 1.472$, $P < 0.02$) (Table S4). SAMOVA results showed that F_{CT} steadily increased as the number of groups of populations increased, from 0.468 for two groups, up to 0.801 for 16 groups (data not shown).

The haplotype network from mtDNA shows relationships that are consistent with those recovered in the phylogenetic analyses; that is, haplotypes from the Northern (Fig. S3a) and Southern clade (Fig. S3b) are more related to those of their own clade and are very distant between clades. Besides that, there is a strong population structure with a large number of mutational steps splitting the main haplotypes, especially in the Northern clade.

The two main clades seem to have diverged at about 362,000 years ago. The TMRCA for North and South haplotypes was estimated at 208,000 and 187,000 years ago, respectively. The subclades have a more recent origin, most of them dating from the upper Pleistocene (Fig. 2). Finally, *A. longirostri sensu stricto* is probably the only well supported clade that comprises individuals from Rolante (type-locality) and Nova Palma.

DISCUSSION

A SPECIES COMPLEX WITHIN *AEGLA LONGIROSTRI*

Our results clearly reject the hypothesis that *A. longirostri* comprises a monophyletic group. We observed the presence of two major clades (North and South) that, although geographically close, are genetically very distinct from each other. These two major clades (also non-monophyletic) are more closely related to other species of aeglids than to each other. Within these major clades several well-supported subclades were recovered. When we consider the tree that includes representatives of the main *Aegla* groups, it is evident that the specimens from the North and South belong to distinct clades according to the phylogenetic classification of Pérez-Losada *et al.*, (2004). Northern sequences belong to clade E, in which *A. longirostri* was originally included, while the Southern sequences belong to clade D. In addition, several

species are inserted between the sequences of the present study, reasserting the polyphyly of *A. longirostri*. Population analyzes also support the existence of several cryptic species, as they indicate the presence of structured and highly differentiated populations or groups of populations. Surprisingly, individuals from the same population (i.e., Soledade) were included in two very distant clades within Northern group (mtDNA divergence of 4.2%), suggesting the presence of more than one cryptic species sympatricaly occurring in the same locality. Nonetheless, intrapopulation genetic variation was generally low.

The species delimitation methods indicated the presence of at least 14 putative cryptic species. Species delimitation models have demonstrated considerable robustness (Fujisawa & Barraclough, 2013); methods such as GMYC and ABGD showed good concordance and produce up to 90% identity between species and MOTUs (Talavera *et al.*, 2013; Pentinsaari, Vos & Mutanen, 2016; Karanovic, Djurakic & Eberhard, 2016). Hence, they are useful to designate an optimal divergence threshold to pinpoint potential cryptic species (Talavera *et al.*, 2013). However, ABGD seems to be sensitive to singleton sequences, while GMYC showed oversplitting tendencies (Pentinsaari *et al.*, 2016). Although robust, ABDG and GMYC entities recovered in this study cannot be directly considered equivalent to 'species', but to 'potential species', and the specific status should be assessed with additional morphological and ecological evidence. Interestingly, BF estimators reinforce these results, since models that suggest the presence of several cryptic species were strongly supported. Despite the considerable high number of potential cryptic species within *A. longirostri*, we argue below that our results are robust and require further attention.

A large number of *Aegla* species are restricted to headwaters (Santos *et al.*, 2015) usually located on hillsides or high altitude regions (Bond-Buckup & Buckup, 1994), suggesting a possible contribution of landscape topography to crab diversification. Indeed, populations from the Northern clade - an area of higher altitudes - seem to be more

differentiated and genetically distant, suggesting a possible influence of geographical physiognomy on the diversification of populations. In a recent paper, Bertuzzo *et al.,* (2016) explained how biodiversity changes with elevation in fluvial landscapes. According to the authors, experimental evidence suggests that biodiversity often peaks at intermediate elevations, but a clear explanation is still elusive. They showed that mountainous landscapes hold fractal properties with elevational bands forming habitat patches that are characterized by different area extent and connectivity, well-known drivers of biodiversity. This strongly suggest that fluvial geomorphology likely has an important, yet thus far overlooked, role in shaping patterns of freshwater species richness. In fact, northeastern Rio Grande do Sul State shelters the highest aeglid richness in Brazil (Pérez-Losada *et al.,* 2009), and an association between altitude and occurrence of aeglids is strong and generally positive (Tumini *et al.,* 2016).

In addition, many biological features of the group could limit gene flow between populations, which increases the probability of speciation events and, consequently, the emergence of cryptic species. First, aeglids are highly demanding in terms of environmental quality. They inhabit particular microhabitats within pristine areas, often in streams of small order, and possess a relatively high metabolism when compared to other decapods, which hints that their distribution is probably restricted by certain environmental conditions (Dalosto & Santos 2011; Zimmermann, Dambros & Santos, 2016). Second, their dispersion capability is probably low and their usual movements are against the direction of flow (Ayres-Peres *et al.,* 2011). Dispersion should thus primarily occur unintentionally, with animals being carried away by water currents during floods. Third, characteristics such as the presence of direct development and parental care (López-Greco *et al.,* 2009) and habitat specialization (Baumart *et al.,* 2015) should further limit the dispersion capabilities of these animals while promoting the isolation and differentiation of populations.

BIOGEOGRAPHY OF *AEGLA LONGIROSTRI* COMPLEX

According to the most accepted hypothesis about the origin of the group, Aeglidae arose in a marine setting from Pacific ancestors during the Late Cretaceous and subsequently adapted and dispersed towards freshwater habitats (Pérez-Losada *et al.*, 2004). Consequently, species located near the Atlantic should be the most recent ones with relatively recent origins. A molecular phylogeny of the group suggests that the current species belong to five clades (A-E): Clades A and B include the Chilean and Southern Argentinean species while clades C, D, and E include the North Argentinean, Uruguayan, and Brazilian species. Although clades D and E showed closer relationships, the relationships among clades C, D, and E were not strongly supported and alternative rearrangements between these clades could not be rejected (Pérez-Losada *et al.*, 2004).

The clear genetic distance between the populations collected in the Meridional Plateau (Northern clade, belonging to clade E of Pérez-Losada *et al.*, 2004) and the Central Depression (Southern clade, belonging to clade D of Pérez-Losada *et al.*, 2004) presented here is remarkable: although geographically close, populations of these two groups are highly differentiated from each other. The Meridional Plateau is characterized by a wavy surface, around 700 meters above sea level (a.s.l.), in which the highest altitudes in the state are found. These high altitudes decrease both eastward and westward. The Central Depression is formed by relatively low, flat or slightly wavy lands between 100 and 200 meters a.s.l. (see Gonçalves & Santos, 1985; Becker & Nunes, 2012 for review). Thus, these altitude differences could have acted as a barrier isolating the populations and preventing their connection. Since these formations precede the origin and diversification of aeglids (dating from the Mesozoic, as well the main rivers that form the river basins of the Rio Grande do Sul state, see Ribeiro, 2006), one hypothesis would be that there were at least two independent events of colonization from the ancestral populations. Furthermore, considering the difference in altitude and that the

Northern clade has the most recent origin (clade E in Pérez-Losada *et al.*, 2004), it would be plausible that this clade was derived from individuals coming from the northern continuation of Meridional Plateau area. Meanwhile, the Southern clade must have originated directly from relatives of Argentina and Uruguay (Pérez-Losada *et al.*, 2004).

Regarding the origin of subclades (i.e., potential cryptic species), most of them had originated during the Late Pleistocene (126,000 - 11,700 BP). Most Quaternary studies in southern Brazil are restricted to the Atlantic Coast, whereas inland areas are poorly studied (Stevaux, 2000). Between the end of the Pleistocene and the Early Holocene (~40,000 – 8,000 BP), the climate was dry. In the upper Paraná River (South Brazil), the sandy gravel facies unit was interpreted as having originated by flash flood and grain flow processes in a typical dry climate braided channel river. Drier conditions were also deduced from the low percentage of organic and savanna pollen content. Then, during the period of 8000 - 3500 years BP, the lake cores displayed an increase in organic material with abundant forest elements and a decrease in floating sand. These facts indicate lesser eolian activity – or more vegetation cover (Stevaux, 2000). Spalding and Lorscheitter (2015) found similar results by analyzing the palynology of the last 34,000 years for a sedimentary profile from the east portion of Rio Grande do Sul state. They observed an alternation between dry and humid phases, with predominance of cold and semi-arid climate during the Late Pleistocene, which must have influenced the population dynamics of freshwater life.

Specifically, aeglid populations were possibly isolated during drier periods, which may have reduced or even restricted the gene flow, a first step towards speciation (Irwin *et al.*, 2005). More recently, when the climate became humid, some of these populations were possibly reconnected, even though they were already reproductively isolated (e.g., populations from Soledade). Indeed, as present-day South American drainage systems were mostly established ~8 Mya (Lundberg, 1998), Pleistocene refuge-allopatric divergence model may only apply to

the shallower nodes of the *Aegla* phylogeny (Pérez-Losada *et al.*, 2004). As these events are relatively recent, phenotypic evolution may not match the faster genetic divergences of mtDNA, explaining the cryptic diversity found within *A. longirostri*, and the absence of significant differences in diagnostic characters. Similar results were observed, for example, for copepods (Rocha-Olivares, Fleeger & Foltz, 2001), freshwater crabs (Daniels *et al.*, 2003), harvestmen (Boyer, Baker & Giribet, 2007) and snails (Moussali & Herbert, 2016), in which large genetic variation was not reflected in the morphology of organisms.

IMPLICATIONS FOR THE SYSTEMATICS AND CONSERVATION OF *AEGLA*

One major obstacle in species delimitation studies is the presence of cryptic lineages. These lineages are difficult to identify morphologically despite the evolutionary processes that have occurred during speciation within the species complex (Bickford *et al.*, 2007; Puckridge *et al.*, 2013). Consequently, identifying species exclusively on the basis of morphological characters can lead to erroneous taxonomic classifications because subtle interspecific morphological differences can easily be overlooked (Colborn *et al.*, 2001). This premise may be especially true for *Aegla* species, and we show here that the real diversity of the group is likely underestimated.

As previously mentioned, diagnostic characters within *Aegla* are very limited and exhibit low taxonomic variation (Bond-Buckup & Buckup, 1994). The subtle phenotypic differences and the problematic distinction between intra- and interspecific variation should generate a discrepancy between the number of species described and the actual number of species, with cryptic diversity probably hidden within this limited morphological differentiation. Therefore, it is of the utmost importance to seek new diagnostic characters and/or techniques (e.g., electron microscopy, morphology of reproductive appendices) for the identification and description of *Aegla* species, including the species in the present study, which

will be formally described in the near future. It should be noted that integrative taxonomy (i.e., integrating information from different types of sources and methodologies) is a good way to improve the quality of species identifications and associated descriptions because it is based in multiple and complementary perspectives (Will, Mishler & Wheeler, 2005; Pante, Schoelinck & Puillandre, 2014).

Based on our results, we predict that more cryptic species complexes occur within *Aegla*. For example, as observed for *A. longirostri* (Marchiori *et al.*, 2014), significant intraspecific morphological differences in shape carapace were also found for other species in the genus. Carapace shapes differed among the river-basin populations of *Aegla plana* Buckup and Rossi, 1977 (Hepp *et al.*, 2012), *A. platensis* Schmitt, 1942 (Marchiori, Fornel & Santos, 2015) and *Aegla schmitti* Hobbs III, 1979 (Trevisan *et al.*, 2016). In all cases, populations are allopatric and/or occur in high-altitude areas, suggesting geographical isolation. Although the morphological variation could respond to the spatial and environmental heterogeneity that exists in the basins examined, there is the possibility that those populations are actually different species. Therefore, a detailed molecular analysis coupled with a taxonomic revision of the group is crucial. As highlighted by Adams *et al.*, (2014), recent estimates of global biodiversity do not formally explore the real "elephant in the room", namely, what proportion of species are taxonomically invisible to conventional assessments, and thus, as undiagnosed cryptic species, remain uncountable until revealed by multi-gene molecular assessments.

A complicating issue is that several of the known *Aegla* species have narrow distributions and are therefore of significant conservation concern. A recent study assessed the conservation status of 77 aeglid species and found that 66% were threatened (Santos *et al.*, 2016), mainly due to their narrow distributions and the rapid degradation of the freshwater habitats they occupy. *Aegla longirostri* was considered as having deficient data for an accurate status conservation assessment due to the evidences for cryptic diversity hidden in this species

(Santos *et al.,* 2016). Even during data collection for this study, some of the sampled populations were located in highly degraded sites with little or no riparian vegetation and surrounded by croplands. Molecular evidence has revealed that several already endangered species are cryptic species complexes, making them a collection of even more critically endangered species with fewer numbers and smaller distributions (Dayrat, 2005). Preventing habitat loss is perhaps the greatest challenge for the conservation of global biodiversity, and prioritizing habitats for conservation often relies on estimation of species richness and endemism. Thus, it is imperative to reveal the cryptic diversity within *Aegla* in order to correctly apply effective management and conservation measures.

ACKNOWLEDGEMENTS

We are grateful to CNPq for productivity fellowship granted to SS. To CAPES for scholarship to MSC and postdoctoral fellowship to BLZ (PNPD – Programa Nacional de Pós Doutorado). Special thanks to Alexandre V. Palaoro and Marcelo M. Dalosto for providing helpful comments and suggestions on the manuscript. We are grateful to CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico for funding this research (Universal – N° 480583/2013-7).

REFERENCES

Adams M, Raadik TA, Burridge CP, Georges A. 2014. Global biodiversity assessment and hyper-cryptic species complexes: more than one species of elephant in the room? *Systematic Biology* **63:** 518-533.

Agapow PM, Bininda-Emonds ORP, Crandall KA, Gittleman JL, Mace GM, Marshall JC, Purvis A. 2004. The impact of species concept on biodiversity studies. *The Quarterly Review of Biology* **79:** 161-179.

Avise JC. 2009. Phylogeography: retrospect and prospect. *Journal of Biogeography* **36:** 3-15.

Ayres-Peres L, Coutinho C, Baumart JS, Gonçalves AS, Araujo PB, Santos S. 2011. Radio-telemetry techniques in the study of displacement of freshwater anomurans. *Nauplius* **19:** 41-54.

Bandelt HJ, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16:** 37-48.

Barber BR, Xu J, Pérez-Losada M, Jara CG, Crandall K.A. 2012. Conflicting evolutionary patterns due to mitochondrial introgression and multilocus phylogeography of the Patagonian freshwater crab *Aegla neuquensis*. *PLoS One* **7:** e37105.

Bartholomei-Santos ML, Roratto PA, Santos S. 2011. High genetic differentiation of *Aegla longirostri* (Crustacea, Decapoda, Anomura) populations in southern Brazil revealed by multi-loci microsatellite analysis. *Genetics and Molecular Research* **10:** 4133-4146.

Baumart JS, Dalosto MM, Gonçalves AS, Palaoro AV, Santos S. 2015. How to deal with a bad neighbor? Strategies of sympatric freshwater decapods (Crustacea) for coexistence. *Hydrobiology* **762:** 29-39.

Becker ES, Nunes PM. 2012. Relevo do Rio Grande do Sul, Brasil, e sua representação em maquete. *Revista Percurso* **4:** 113-132.

Bergsten J, Nilsson AN, Ronquist F. 2013. Bayesian tests of topology hypotheses with an example from diving beetles. *Systematic Biology* **62:** 660-673.

Bertuzzo E, Carrara F, Mari L, Altermatt F, Rodriguez-Iturbe I, Rinaldo A. 2016. Geomorphic controls on elevational gradients of species richness. *Proceedings of the National Academy of Sciences of the United States of America* **113:** 1737-1742.

Bickford D, Lohman DJ, Sodhi NS, Ng PK, Meier R, Winker K, Ingram KK, Das I. 2007. Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution* **22:** 148-155.

Bisby FA, Coddington J, Thorpe JP. 1995 Characterization of biodiversity. *Global biodiversity assessment* **162:** 14624-14627.

Bond-Buckup G, Buckup L. 1994. A família Aeglidae (Crustacea, Decapoda, Anomura). *Arquivos de Zoologia* **32:** 159-347.

Boyer SL, Baker JM, Giribet G. 2007 Deep genetic divergences in *Aoraki denticulata* (Arachnida, Opiliones, Cyphophthalmi): a widespread ‘mite harvestman’defies DNA taxonomy. *Molecular Ecology* **16:** 4999-5016.

Bracken-Grissom HD, Cannon ME, Cabezas P, Feldmann RM, Schweitzer CE, Ahyong ST, Felder DL, Lemaitre R, Crandall KA. 2013. A comprehensive and integrative reconstruction of evolutionary history for Anomura (Crustacea: Decapoda). *BMC Evolutionary Biology* **13:** 1.

Bracken-Grissom HD, Felder DL, Vollmer NL, Martin JW, Crandall KA. 2012. Phylogenetics links monster larva to deep-sea shrimp. *Ecology and Evolution* **2:** 2367-2373.

Choi SC. 2016. Methods for delimiting species via population genetics and phylogenetics using genotype data. *Genes Genomics* **38:** 905-915.

Colborn J, Crabtree RE, Shaklee JB, Pfeiler E, Bowen BW. 2001. The evolutionary enigma of bonefishes (Albula spp.): cryptic species and ancient separations in a globally distributed shorefish. *Evolution* **55:** 807-820.

Dalosto M, Santos S. 2011. Differences in oxygen consumption and diel activity as adaptations related to microhabitat in Neotropical freshwater decapods (Crustacea). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **160:** 461–46.

Daniels SR, Gouws G, Stewart BA, Coke M. 2003. Molecular and morphometric data demonstrate the presence of cryptic lineages among freshwater crabs (Decapoda: Potamonautidae: Potamonautes) from the Drakensberg Mountains, South Africa. *Biological Journal of the Linnean Society* **78:** 129-147.

Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9:** 772.

Dayrat B. 2005. Towards integrative taxonomy. *Biological Journal of the Linnean Society* **85:** 407-415.

Drummond AJ, Suchard MA, Xie D, Rambaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* **29:** 1969-1973.

Dupanloup I, Schneider S, Excoffier L. 2002. A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology* **11:** 2571-81.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids Research* **32:** 1792-1797.

Excoffier L, Laval G, Schneider S. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1:** 47-50.

Flot JF. 2015. Species Delimitation's Coming of Age. *Systematic Biology* **64:** 897–899.

Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147:** 915-925.

Fu YX, Li WH. 1993. Statistical tests of neutrality of mutations. *Genetics* **133**, 693-709.

Fujisawa T, Barraclough TG. 2013. Delimiting species using single-locus data and the Generalized Mixed Yule Coalescent (GMYC) approach: a revised method and evaluation on simulated datasets. *Systematic Biology* **62:** 707–724.

Gonçalves JMS, Santos NM. 1985. Análise das classificações do relevo para o Rio Grande do Sul. *Boletim Gaúcho de Geografia* **13:** 3-20.

Grummer JA, Bryson RW, Reeder TW. 2014. Species delimitation using Bayes factors: simulations and application to the Sceloporus scalaris species group (Squamata: Phrynosomatidae). *Systematic Biology* **63:** 119-133.

Hepp LU, Fornel R, Restello RM, Trevisan A, Santos S. 2012. Intraspecific morphological variation in a freshwater crustacean *Aegla plana* in Southern Brazil: effects of geographical isolation on carapace shape. *Journal of Crustacean Biology* **32:** 511-518.

Hickerson MJ, Carstens BC, Cavender-Bares J, Crandall KA, Graham CG, Johnson JB, Rissler L, Victoriano PF, Yoder AD. 2010. Phylogeography's past, present, and future: 10 years after Avise, 2000. *Molecular Phylogenetics and Evolution* **54:** 291-301.

Irwin DE, Bensch S, Irwin JH, Price TD. 2005. Speciation by distance in a ring species. *Science* **307:** 414-416.

Jara CG, Perez-Losada M, Crandall KA. 2003. A new species of freshwater anomuran crab of the genus *Aegla* Leach, 1821 (Crustacea: Decapoda: Aeglidae) from the Nahuelbuta Coastal Range, Chile. *Proceedings of the Biological Society of Washington* **116:** 933-942.

Karanovic T, Djurakic M, Eberhard SM. 2016. Cryptic species or inadequate taxonomy? Implementation of 2D geometric morphometrics based on integumental organs as landmarks for delimitation and description of copepod taxa. *Systematic Biology* **65:** 304-327.

Kass RE, Raftery AE. 1995. Bayes factors. *Journal of the American statistical association* **90:** 773-795.

Lefébure T, Douady CJ, Gouy M, Gibert J. 2006. Relationship between morphological taxonomy and molecular divergence within Crustacea: proposal of a molecular threshold to help species delimitation. *Molecular Phylogenetics and Evolution* **40:** 435-447.

López-Greco LS, Fransozo V, Negreiros-Fransozo ML, Dos Santos DC. 2009. Comparative morphology of the seminal receptacles of *Ocypode quadrata* (Fabricius, 1787) (Brachyura, Ocypodoidea). *Zootaxa* **2106:** 41-50.

Lundberg JG. 1998. The temporal context for the diversification of Neotropical fishes. *Phylogeny and classification of Neotropical fishes* 49-68.

Marchiori AB, Bartholomei-Santos ML, Santos S. 2014. Intraspecific variation in *Aegla longirostri* (Crustacea: Decapoda: Anomura) revealed by geometric morphometrics: evidence for ongoing speciation? *Biological Journal of the Linnean Society* **112:** 31-39.

Marchiori AB, Fornel R, Santos S. 2015. Morphometric variation in allopatric populations of *Aegla platensis* (Crustacea: Decapoda: Anomura): possible evidence for cryptic speciation. *Zoomorphology* **134:** 45-53.

Martin JW, Crandall KA, Felder DL. 2009. Decapod crustacean phylogenetics. Boca Raton, London, New York: CRC Press, Taylor & Francis Group. 616 p.

Moussalli A, Herbert DG. 2016. Deep molecular divergence and exceptional morphological stasis in dwarf cannibal snails Nata sensu lato Watson, 1934 (Rhytididae) of southern Africa. *Molecular Phylogenetics and Evolution* **95:** 100-115.

Orme CDL, Davies RG, Burgess M, Eigenbrod F, Pickup N, Olson VA, Webster AJ, Ding T, Rasmussen PC, Ridgely RS, Stattersfield AJ, Bennett PM, Blackburn TM, Gaston KJ, Owens PF. 2005. Global hotspots of species richness are not congruent with endemism or threat. *Nature* **436:** 1016–1019.

Padial JM, Miralles A, De la Riva I, Vences M. 2010. The integrative future of taxonomy. *Frontiers in Zoology* **7:** 1.

Page TJ, Hughes JM. 2007. Radically different scales of phylogeographic structuring within cryptic species of freshwater shrimp (Atyidae: Caridina). *Limnology and Oceanography* **52:** 1055-1066.

Pante E, Schoelinck C, Puillandre N. 2014. From integrative taxonomy to species description: one step beyond. *Systematic Biology* **64:** 152-160.

Pentinsaari M, Vos R, Mutanen M. 2016. Algorithmic single-locus species delimitation: effects of sampling effort, variation and nonmonophyly in four methods and 1870 species of beetles. *Molecular Ecology Resources*.

Pérez-Losada M, Bond-Buckup G, Jara CG, Crandall KA. 2004. Molecular systematics and biogeography of the southern South American freshwater "crabs" *Aegla* (Decapoda: Anomura: Aeglidae) using multiple heuristic tree search approaches. *Systematic Biology* **53**: 767-780.

Pérez-Losada M, Bond-Buckup G, Jara CG, Crandall KA. 2009. Conservation assessment of southern South American freshwater ecoregions on the basis of the distribution and genetic diversity of crabs from the genus *Aegla*. *Conservation Biology* **23:** 692-702.

Pérez-Losada M, Jara CG, Bond-Buckup G, Crandall KA. 2002. Conservation phylogenetics of Chilean freshwater crabs *Aegla* (Anomura, Aeglidae): assigning priorities for aquatic habitat protection. *Biological Conservation* **105:** 345-353.

Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S, Kamoun S, Sumlin WD, Vogler AP. 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology* **55:** 595-609.

Puckridge M, Andreakis N, Appleyard SA, Ward RD. 2013. Cryptic diversity in flathead fishes (Scorpaeniformes: Platycephalidae) across the Indo-West Pacific uncovered by DNA barcoding. *Molecular Ecology Resources* **13:** 32-42.

Puillandre N, Lambert A, Brouillet S, Achaz G. 2012. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* **21:** 1864-1877.

Puillandre N, Macpherson E, Lambourdière J, Cruaud C, Boisselier-Dubayle MC, Samadi S. 2011. Barcoding type specimens helps to identify synonyms and an unnamed new

species in Eumunida Smith, 1883 (Decapoda: Eumunididae). *Invertebrate Systematics* **25:** 322-333.

R Development Core Team. 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Rambaut A. 2014. Figtree v.1.4.2. Software. Institute of Evolutionary Biology, Univ. Edinburgh. Available from: URL http://tree.bio.ed.ac.uk/software/figtree/

Rambaut A, Suchard MA, Xie D, Drummond AJ. 2014. Tracer v1.6. Software. Institute of Evolutionary Biology, Univ. Edinburgh. Available from: URL http://beast.bio.ed.ac.uk/Tracer.

Ribeiro AC. 2006. Tectonic history and the biogeography of the freshwater fishes from the coastal drainages of eastern Brazil: an example of faunal evolution associated with a divergent continental margin. *Neotropical Ichthyology* **4:** 225-246.

Riddle BR, Hafner DJ. 2006. A step-wise approach to integrating phylogeographic and phylogenetic biogeographic perspectives on the history of a core North American warm deserts biota. *Journal of Arid Environments* **66:** 435-461.

Rissler LJ, Apodaca JJ. 2007 Adding more ecology into species delimitation: ecological niche models and phylogeography help define cryptic species in the black salamander (Aneides flavipunctatus). *Systematic Biology* **56:** 924-942.

Rocha-Olivares A, Fleeger JW, Foltz DW. 2001. Decoupling of molecular and morphological evolution in deep lineages of a meiobenthic harpacticoid copepod. *Molecular Biology and Evolution* **18:** 1088-1102.

Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**: 2496-2497.

Santos S, Bond-Buckup G, Buckup L, Bartholomei-Santos ML, Pérez-Losada M, Jara CG, Crandall KA. 2015. Three new species of Aeglidae (*Aegla* Leach, 1820) from Paraná State, Brazil. *Journal of Crustacean Biology* **35:** 839-849.

Santos S, Bond-Buckup G, Gonçalves, AS, Bartholomei-Santos ML, Buckup L, Jara CG. 2016. Diversity and conservation status of *Aegla* spp. (Anomura, Aeglidae): an update. *Nauplius* X:000-000.

Santos S, Jara CG, Bartholomei-Santos ML, Pérez-Losada M, Crandall KA. 2013. New species and records of the genus *Aegla* Leach, 1820 (Crustacea, Anomura, Aeglidae) from the west-central region of Rio Grande do Sul, Brazil. *Nauplius* **21:** 211-223.

Silvestro D, Michalak I. 2012. raxmlGUI: a graphical front-end for RAxML. *Organisms Diversity Evolutio,* **12:** 335-337.

Simmons MP, Ochoterena H, Carr TG. 2001. Incorporation, relative homoplasy, and effect of gap characters in sequence-based phylogenetic analyses. *Systematic Biology* **50:** 454-462.

Spalding BBC, Lorscheitter ML. 2015. Dry and humid phases in the highlands of southern Brazil during the last 34,000 years, and their influence on the paleoenvironments of the region. *Quaternary International* **377:** 102-111.

Stevaux JC. 2000. Climatic events during the late Pleistocene and Holocene in the upper Parana River: Correlation with NE Argentina and South-Central Brazil. *Quaternary International* **72:** 73-85.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123:** 585-595.

Talavera G, Dincă V, Vila R. 2013. Factors affecting species delimitations with the GMYC model: insights from a butterfly survey. *Methods in Ecology and Evolution* **4:** 1101-1110.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30:** 2725-2729.

Teske PR, Beheregaray LB. 2009. Intron-spanning primers for the amplification of the nuclear ANT gene in decapod crustaceans. *Molecular Ecology Resources* **9:** 774-776.

Trevisan A, Marochi MZ, Costa M, Santos S, Masunari S. 2016. Effects of the evolution of the Serra do Mar mountains on the shape of the geographically isolated populations of *Aegla schmitti* Hobbs III, 1979 (Decapoda: Anomura). *Acta Zoologica* **97:** 34–41.

Tumini G, Giri F, Williner V, Collins PA. 2016. The importance of biogeographical history and extant environmental conditions as drivers of freshwater decapod distribution in southern South America. *Freshwater Biology* **61:** 715-728.

Will KW, Mishler BD, Wheeler QD. 2005. The perils of DNA barcoding and the need for integrative taxonomy. *Systematic Biology* **54:** 844-851.

Xiao JH, Wan NX, Li YW, Murphy RW, Wan DG, Niu LM, Hu HY, Fu YG, Huang DW. 2010. Molecular approaches to identify cryptic species and polymorphic species within a complex community of fig wasps. *PLoS One* **5:** e15067.

Xie W, Lewis PO, Fan Y, Kuo L, Chen M-H. 2011. Improvingmarginal likelihood estimation for bayesian phylogenetic model selection. *Systematic Biology* **60:** 150-160.

Xu J, Pérez, Losada M, Jara CG, Crandall K.A. 2009. Pleistocene glaciation leaves deep signature on the freshwater crab *Aegla alacalufi* in Chilean Patagonia. *Molecular Ecology* **18:** 904-918.

Yang Z, Rannala B. 2010. Bayesian species delimitation using multilocus sequence data. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 9264-9269.

Yang Z, Rannala B. 2014. Unguided species delimitation using DNA sequence data from multiple loci. *Molecular Biology and Evolution* **31:** 3125-3135.

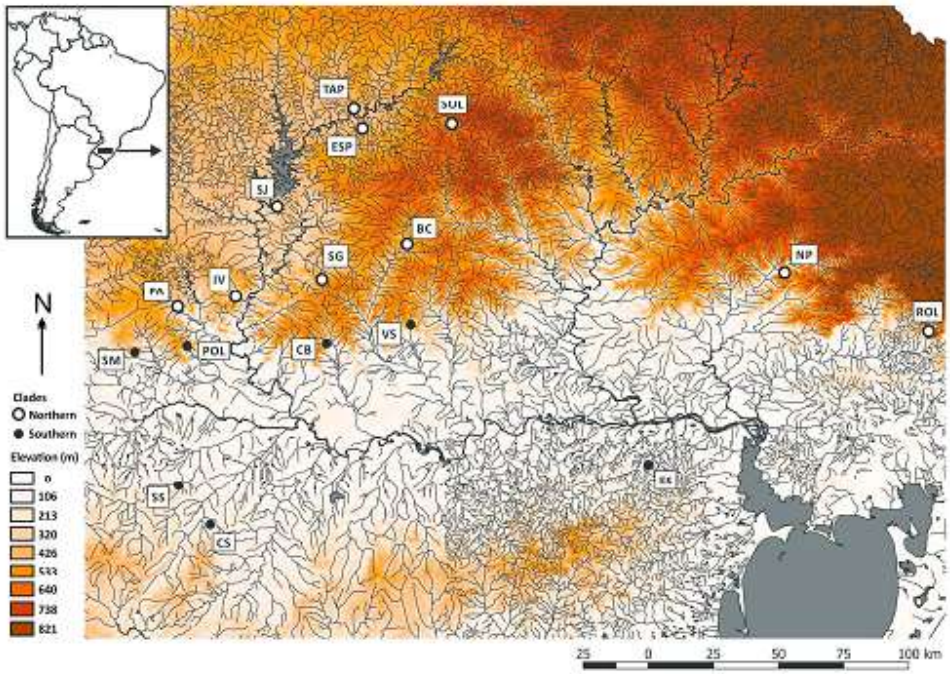
Zimmermann BL, Dambros CS, Santos S. 2016. Association of microhabitat variables with the abundance and distribution of two neotropical freshwater decapods (Anomura: Brachyura). *Journal of the Crustacean Biology* **36:** 198-204.

FIGURE LEGENDS

Figure 1. Map showing the sampling locations for "*Aegla longirostri"* in the Rio Grande do Sul State. White circles represent populations sampled in the Meridional Plateau (Northern Clade). Black circles represent populations sampled in the Central Depression (Southern Clade). BC: Barros Cassal; CB: Cerro Branco; CS: Caçapava do Sul; ES: Eldorado do Sul; ESP: Espumoso; IV: Ivorá; NP: Nova Petrópolis; PA: Nova Palma; ROL: Rolante; SG: Segredo; SJ: Salto do Jacuí; SM: Santa Maria; SOL: Soledade; SS: São Sepé; TAP: Tapera; POL: São João do Polêsine; VS: Vale do Sol.

Figure 2. Bayesian tree based on mtDNA haplotypes of "*Aegla longirostri"* using the HKY+G+I substitution model (considering gaps and missing data). Numbers above branches are Bayesian posterior probabilities. Numbers below branches are the estimates of mean divergence time [thousand years ago (95% HPD intervals)]. Molecular entities delimited by ABDG and GMYC algorithms are also represented in the tree. Dotted square represents *A. longirostri sensu stricto*. BC: Barros Cassal; CB: Cerro Branco; CS: Caçapava do Sul; ES: Eldorado do Sul; ESP: Espumoso; IV: Ivorá; NP: Nova Petrópolis; PA: Nova Palma; ROL: Rolante; SG: Segredo; SJ: Salto do Jacuí; SM: Santa Maria; SOL: Soledade; SS: São Sepé; TAP: Tapera; POL: São João do Polêsine; VS: Vale do Sol.

Figure 3. Bayesian tree based on concatenated sequences of "*Aegla longirostri"* (mtDNA + nucDNA) using the GTR+G+I substitution model (considering gaps and missing data). Numbers above branches are Bayesian posterior probabilities. BC: Barros Cassal; CB: Cerro Branco; CS: Caçapava do Sul; ES: Eldorado do Sul; ESP: Espumoso; IV: Ivorá; NP: Nova Petrópolis; PA: Nova Palma; ROL: Rolante; SG: Segredo; SJ: Salto do Jacuí; SM: Santa Maria; SOL: Soledade; SS: São Sepé; TAP: Tapera; POL: São João do Polêsine; VS: Vale do Sol.



Map showing the sampling locations for "Aegla longirostri" in the Rio Grande do Sul State. White circles represent populations sampled in the Meridional Plateau (Northern Clade). Black circles represent populations sampled in the Central Depression (Southern Clade). BC: Barros Cassal; CB: Cerro Branco; CS: Caçapava do Sul; ES: Eldorado do Sul; ESP: Espumoso; IV: Ivorá; NP: Nova Petrópolis; PA: Nova Palma; ROL: Rolante; SG: Segredo; SJ: Salto do Jacuí; SM: Santa Maria; SOL: Soledade; SS: São Sepé; TAP: Tapera; POL: São João do Polêsine; VS: Vale do Sol.

Figure 1

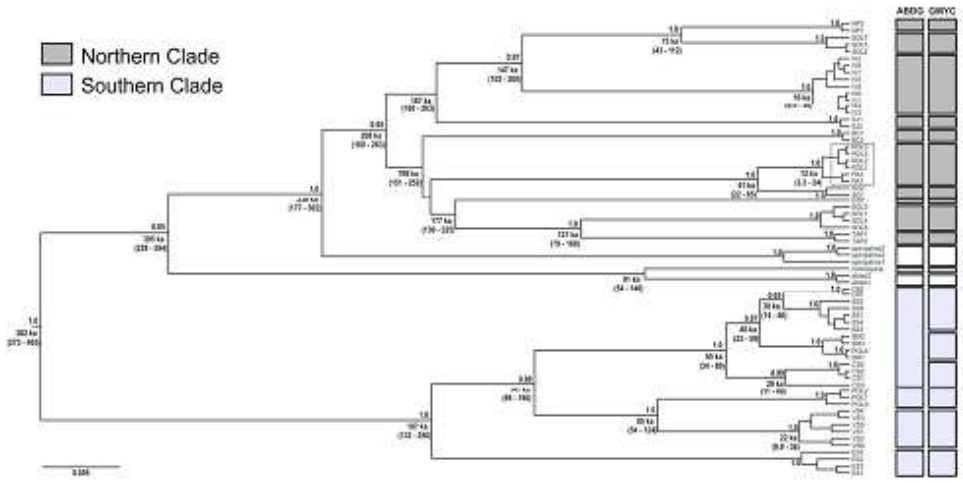
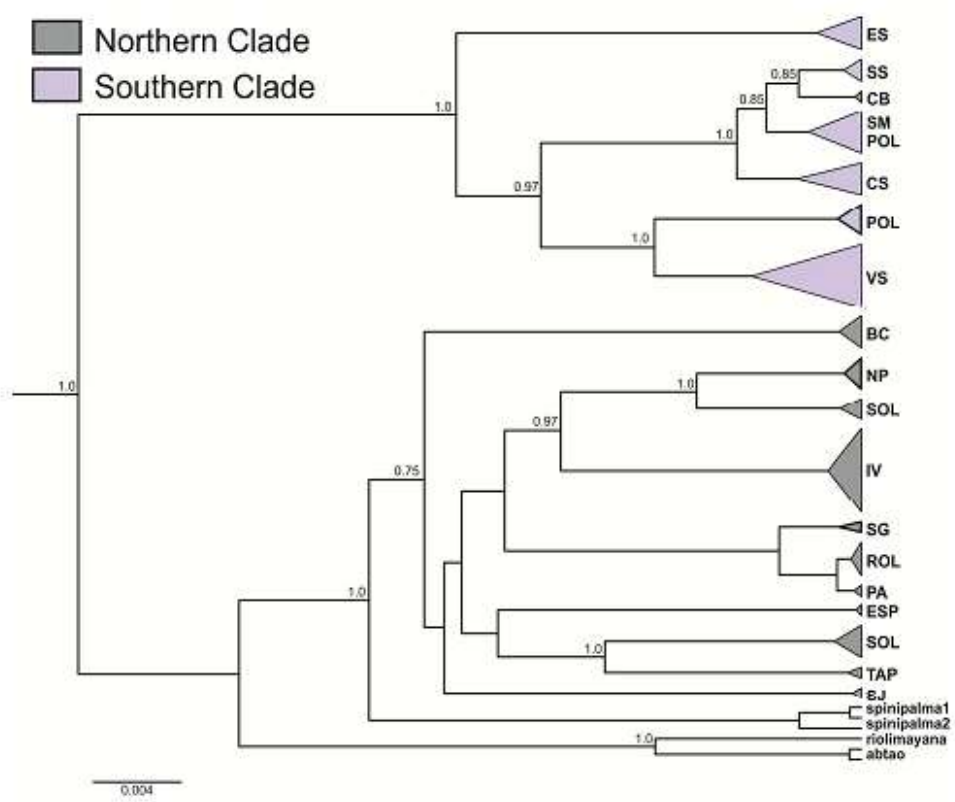


Figure 2. Bayesian tree based on mtDNA haplotypes of "Aegla longirostri" using the HKY+G+I substitution model (considering gaps and missing data). Numbers above branches are Bayesian posterior probabilities. Numbers below branches are the estimates of mean divergence time [thousand years ago (95% HPD intervals)]. Molecular entities delimited by ABDG and GMYC algorithms are also represented in the tree. Dotted square represents A. longirostri sensu stricto. BC: Barros Cassal; CB: Cerro Branco; CS: Caçapava do Sul; ES: Eldorado do Sul; ESP: Espumoso; IV: Ivorá; NP: Nova Petrópolis; PA: Nova Palma; ROL: Rolante; SG: Segredo; SJ: Salto do Jacuí; SM: Santa Maria; SOL: Soledade; SS: São Sepé; TAP: Tapera; POL: São João do Polêsine; VS: Vale do Sol.



Bayesian tree based on concatenated sequences of "Aegla longirostri" (mtDNA + nucDNA) using the GTR+G+I substitution model (considering gaps and missing data). Numbers above branches are Bayesian posterior probabilities. BC: Barros Cassal; CB: Cerro Branco; CS: Caçapava do Sul; ES: Eldorado do Sul; ESP: Espumoso; IV: Ivorá; NP: Nova Petrópolis; PA: Nova Palma; ROL: Rolante; SG: Segredo; SJ: Salto do Jacuí; SM: Santa Maria; SOL: Soledade; SS: São Sepé; TAP: Tapera; POL: São João do Polêsine; VS: Vale do Sol.

Figure 3

TABLES

Table 1. Number of specimens (N), Number of haplotypes (Nh), haplotype diversity (h) and nucleotide diversity (π) for the mtDNA of populations examined in this study.

Locality	**Code**	**N**	**Nh**	**h**	π	**Latitude (S)**	**Longitude (W)**
Barros Cassal	BC	13	2	0.15385	-	29°13'40.82"	52°38'56.51"
Caçapava do Sul	CS	13	3	0.47273	0.00174	30°21'24.51"	53°26'24.33"
Cerro Branco	CB	6	1	-	-	29°37'44.73"	52°58'22.39"
Eldorado do Sul	ES	15	4	0.67949	0.00220	30° 6'46.60"	51°40'53.80"
Espumoso	ESP	15	1	-	-	28°45'46.88"	52°49'43.46"
Ivorá	IV	14	4	0.59091	0.00065	29°29'0.80"	53°34'13.00"
Nova Palma	PA	2	1	-	-	29°26'34.00"	53°20'15.00"
Nova Petrópolis	NP	7	2	0.57143	-	29°20'32.00"	51°08'04.00"
Rolante	ROL	10	1	-	-	29°34'49.36"	50°33'22.28"
Salto do Jacuí	SJ	8	1	-	-	29°04'41.00"	53°10'15.00"
Santa Maria	SM	14	3	0.56364	0.00077	29°40'13.00"	53°45'44.00"
São João do Polênise	POL	15	4	0.61538	0.00828	29°39'0.70"	53°31'13.00"
São Sepé	SS	12	5	0.45455	0.00044	30°11'53.19"	53°34'02.46"
Segredo	SG	15	2	0.43956	0.00078	29°22'11.00"	52°59'29.00"
Soledade	SOL	13	7	0.84615	0.02176	28°44'40.40"	52°28'12.62"
Tapera	TAP	2	2	1.00000	0.00088	28°41'4.07"	52°51'46.67"
Vale do Sol	VS	12	6	0.952	0.00244	29°33'16.97"	52°38'07.22"

Supporting Information

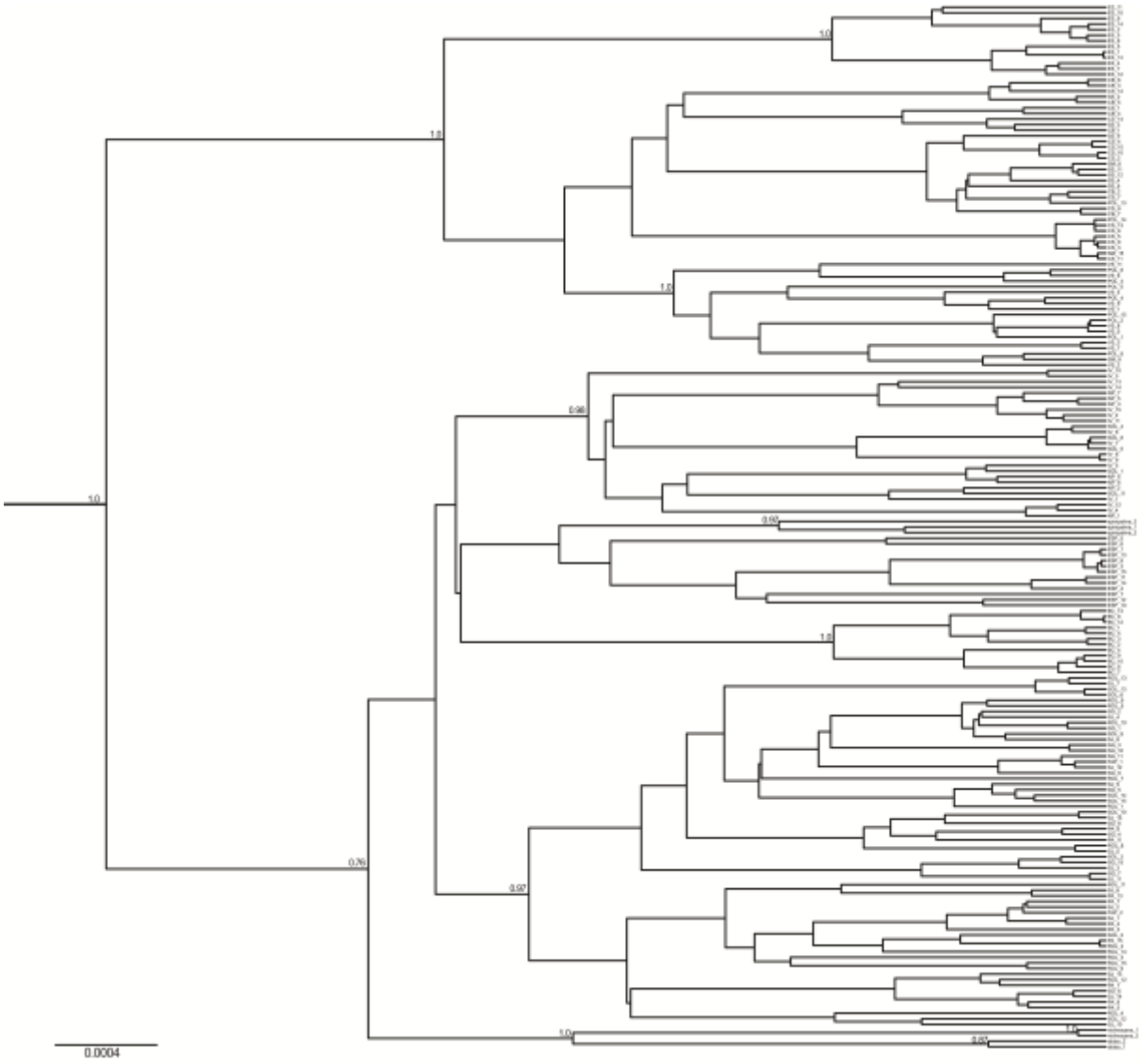


Figure S1. Bayesian tree based on nuclear ANT sequences of "*Aegla longirostri"* using the HKY substitution model (considering gaps and missing data). Numbers above branches are Bayesian posterior probabilities. BC: Barros Cassal; CB: Cerro Branco; CS: Caçapava do Sul; ES: Eldorado do Sul; ESP: Espumoso; IV: Ivorá; NP: Nova Petrópolis; PA: Nova Palma; ROL: Rolante; SG: Segredo; SJ: Salto do Jacuí; SM: Santa Maria; SOL: Soledade; SS: São Sepé; TAP: Tapera; POL: São João do Polêsine; VS: Vale do Sol.

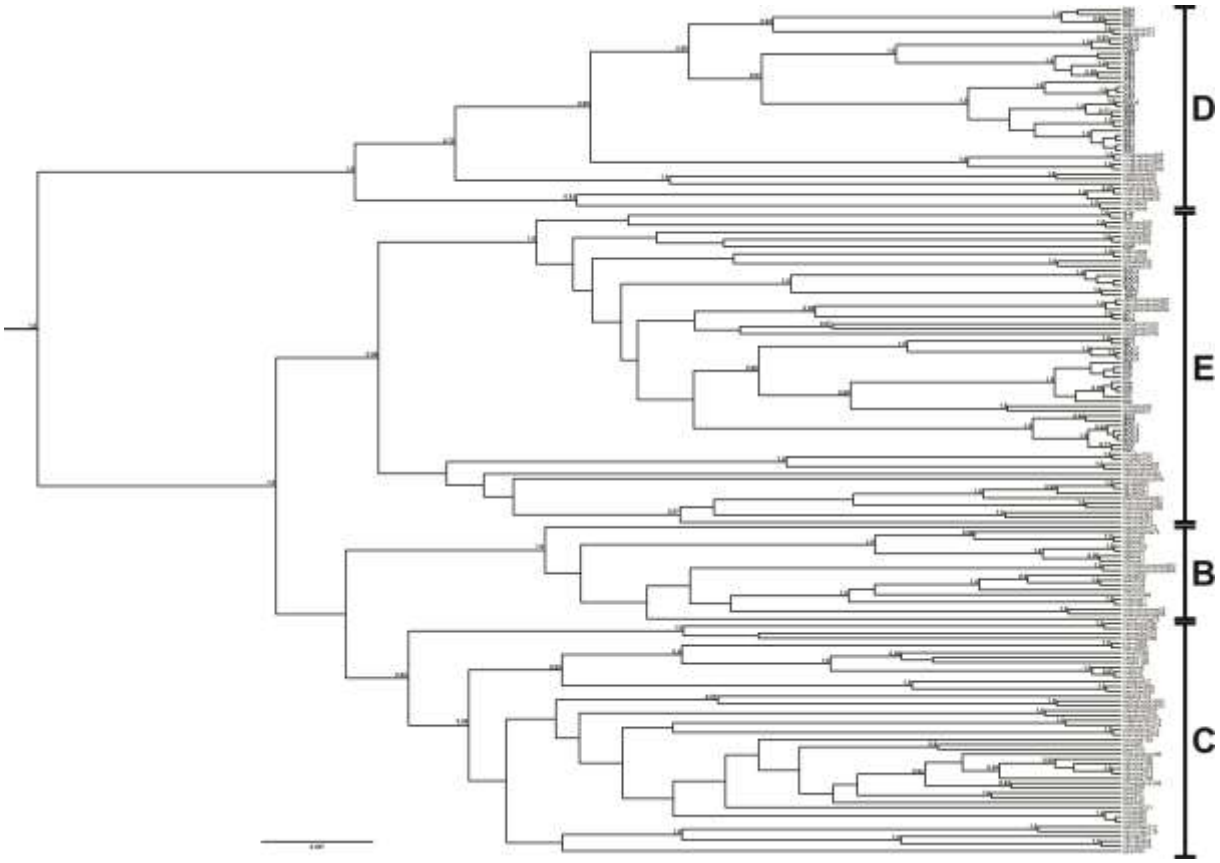


Figure S2. Bayesian tree based on mtDNA sequences (COI + 16S) of *Aegla* from this study and from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), highlighting clades defined by Pérez-Losada et al. (2004). Numbers above branches are Bayesian posterior probabilities. BC: Barros Cassal; CB: Cerro Branco; CS: Caçapava do Sul; ES: Eldorado do Sul; ESP: Espumoso; IV: Ivorá; NP: Nova Petrópolis; PA: Nova Palma; ROL: Rolante; SG: Segredo; SJ: Salto do Jacuí; SM: Santa Maria; SOL: Soledade; SS: São Sepé; TAP: Tapera; POL: São João do Polêsine; VS: Vale do Sol.

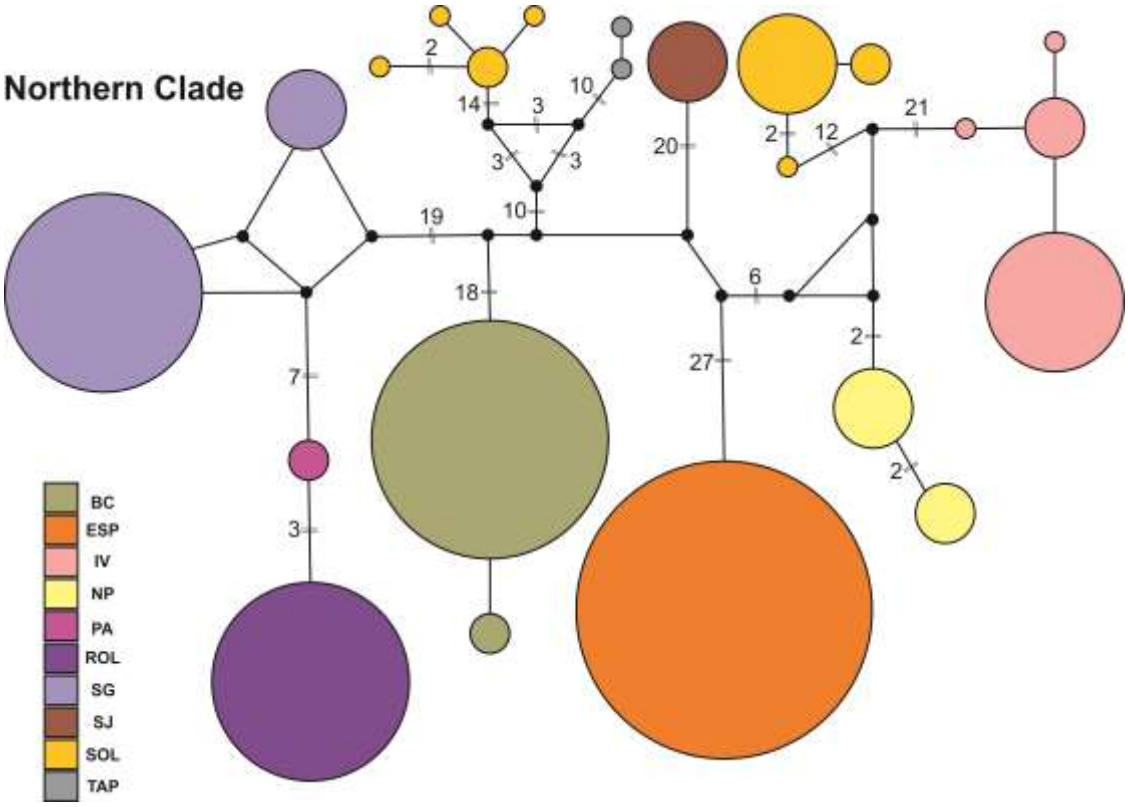


Figure S3. (A) Median-joining haplotype network for mtDNA sequences from Northern Clade. Area of the circles is proportional to the number of individuals of each haplotype found. Black dots represent missing, probably unsampled, haplotypes or extinct lineages. Lines between circles represent the number of mutational steps. No number was indicated when there was only one mutational step between haplotypes. BC: Barros Cassal; ESP: Espumoso; IV: Ivorá; NP: Nova Petrópolis; PA: Nova Palma; ROL: Rolante; SG: Segredo; SJ: Salto do Jacuí; SOL: Soledade; TAP: Tapera.

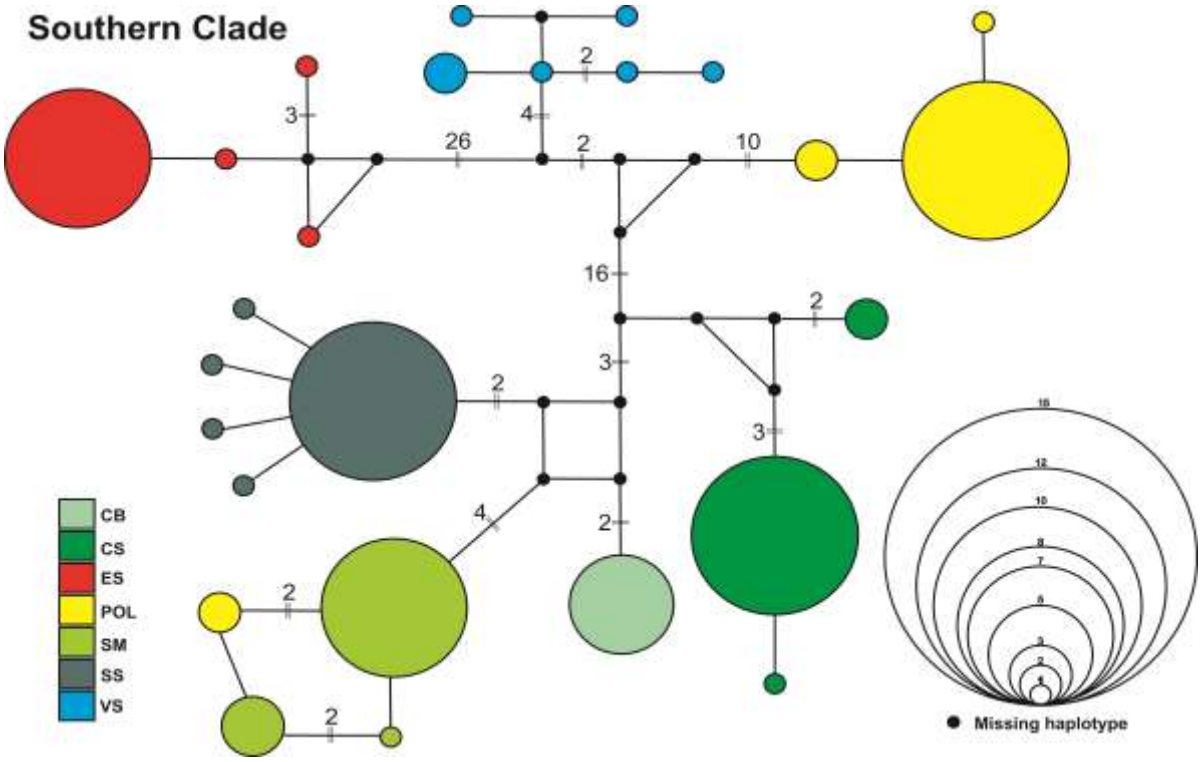


Figure S3. (B) Median-joining haplotype network for mtDNA sequences from Southern Clade. Black dots represent missing, probably unsampled, haplotypes or extinct lineages. Lines between circles represent the number of mutational steps. No number was indicated when there was only one mutational step between haplotypes. CB: Cerro Branco; CS: Caçapava do Sul; ES: Eldorado do Sul; POL: São João do Polêsine; SM: Santa Maria; SS: São Sepé; VS: Vale do Sol.

Table S1. Species delimitation results using Bayes Factor (BF) model selection approaches. Estimations of the marginal likelihood were calculated using stepping-stone sampling runs (SS).

		2*ln*BF	2*ln*BF	2*ln*BF	2*ln*BF	2*ln*BF
		Model 2 *vs*	Model 1 *vs*	Model 1 *vs*	Model 5 *vs*	Model 5 *vs*
	SS	Model 1	Model 3	Model 4	Model 3	Model 4
Model 1	-3931.69					
Model 2	-3935.25					
Model 3	-3797.40					
Model 4	-3784.36					
Model 5	-3869.10					
		7.12	268.58	294.66	143.40	169.48

Table S2. Pairwise F_{ST} estimates among "*Aegla longirostri"* populations. Underlined values are nonsignificant (P > 0.05). All other F_{ST} values are significant at $P < 0.05$. BC: Barros Cassal; CB: Cerro Branco; CS: Caçapava do Sul; ES: Eldorado do Sul; ESP: Espumoso; IV: Ivorá; NP: Nova Petrópolis; PA: Nova Palma; ROL: Rolante; SG: Segredo; SJ: Salto do Jacuí; SM: Santa Maria; SOL: Soledade; SS: São Sepé; TAP: Tapera; POL: São João do Polêsine; VS: Vale do Sol.

	PA	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
ROL	0.7416															
VS	0.9324	0.9814														
BC	0.9662	0.9977	0.9794													
ESP	0.9785	1	0.9868	0.9982												
IV	0.9097	0.9668	0.9547	0.9651	0.9721											
NP	0.9288	0.9934	0.9712	0.9906	0.9952	0.9317										
SG	0.8270	0.9472	0.9750	0.9863	0.9903	0.9581	0.9771									
SJ	0.7641	0.9440	0.9056	0.9280	0.953	0.870	0.8745	0.9332								
SM	0.9563	0.9870	0.9352	0.9871	0.9904	0.9655	0.9794	0.9799	0.9365							
SOL	0.4725	0.6813	0.7322	0.6792	0.7473	0.6078	0.3955	0.6944	0.4694	0.7693						
TAP	0.8845	0.9978	0.9586	0.9939	0.9983	0.9265	0.9807	0.9789	0.8027	0.9771	0.4491					
POL	0.8296	0.9073	0.6753	0.9084	0.9326	0.8893	0.8876	0.9130	0.8076	0.7849	0.7237	0.8576				
CB	0.9565	1	0.9403	0.9979	1	0.9688	0.9935	0.9876	0.9221	0.8785	0.7105	0.9966	0.7438			
ES	0.9418	0.9760	0.9243	0.9749	0.9813	0.9560	0.966	0.9722	0.9111	0.9513	0.7790	0.9611	0.8346	0.9535		
SS	0.9689	0.9934	0.9509	0.9927	0.9949	0.9736	0.9879	0.9859	0.9500	0.8766	0.7840	0.9885	0.7938	0.9217	0.9596	
CS	0.9472	0.9811	0.9182	0.9813	0.9852	0.9609	0.9714	0.9751	0.9238	0.8614	0.7626	0.9663	0.7787	0.8614	0.9403	0.8757

Table S3. Average pairwise differences among and within populations. Below diagonal: average number of pairwise differences between populations. Diagonal elements (bold): average number of pairwise differences within populations. BC: Barros Cassal; CB: Cerro Branco; CS: Caçapava do Sul; ES: Eldorado do Sul; ESP: Espumoso; IV: Ivorá; NP: Nova Petrópolis; PA: Nova Palma; ROL: Rolante; SG: Segredo; SJ: Salto do Jacuí; SM: Santa Maria; SOL: Soledade; SS: São Sepé; TAP: Tapera; POL: São João do Polêsine; VS: Vale do Sol.

	PA	ROL	VS	BC	ESP	NP	IV	SG	SM	SJ	SOL	TAP	POL	CB	ES	SS	CS
PA	**0**																
ROL	0.001	**0**															
VS	0.045	0.046	**0.002**														
BC	0.027	0.028	0.040	**0**													
ESP	0.034	0.035	0.049	0.031	**0**												
NP	0.025	0.026	0.047	0.027	0.029	**0**											
IV	0.028	0.029	0.044	0.027	0.030	0.018	**0***										
SG	0.005	0.006	0.046	0.029	0.034	0.025	0.028	**0***									
SM	0.043	0.044	0.026	0.045	0.047	0.042	0.043	0.043	**0***								
SJ	0.024	0.025	0.036	0.017	0.023	0.018	0.018	0.024	0.035	**0**							
SOL	0.032	0.033	0.050	0.033	0.038	0.021	0.026	0.032	0.049	0.028	**0.021**						
TAP	0.033	0.034	0.048	0.029	0.030	0.027	0.025	0.033	0.046	0.024	0.030	**0***					
POL	0.041	0.042	0.014	0.038	0.048	0.042	0.040	0.042	0.021	0.032	0.046	0.044	**0.007**				
CB	0.042	0.043	0.023	0.044	0.044	0.041	0.044	0.041	0.005	0.032	0.048	0.045	0.019	**0**			
ES	0.042	0.043	0.023	0.042	0.046	0.042	0.041	0.043	0.029	0.032	0.049	0.045	0.024	0.027	**0.002**		
SS	0.044	0.045	0.024	0.044	0.046	0.041	0.046	0.043	0.005	0.034	0.048	0.047	0.020	0.004	0.027	**0***	
CS	0.045	0.046	0.026	0.046	0.046	0.044	0.046	0.045	0.010	0.035	0.050	0.048	0.020	0.008	0.028	0.006	**0.002**

Table S4. Neutrality tests (Fu's *Fs*, Tajima's *D* and Fu and Li's *D*) for the mtDNA of populations examined in this study. BC: Barros Cassal; CB: Cerro Branco; CS: Caçapava do Sul; ES: Eldorado do Sul; ESP: Espumoso; IV: Ivorá; NP: Nova Petrópolis; PA: Nova Palma; ROL: Rolante; SG: Segredo; SJ: Salto do Jacuí; SM: Santa Maria; SOL: Soledade; SS: São Sepé; TAP: Tapera; POL: São João do Polêsine; VS: Vale do Sol. **P* < 0.05, ***P* < 0.01.

Locality	**Fu's *Fs***	**Tajima's *D***	**Fu and Li's *D***
Barros Cassal (BC)	-0.537	-	-
Caçapava do Sul (CS)	0.932	-0.164	1.326
Cerro Branco (CB)	-	-	-
Eldorado do sul (ES)	1.633	1.057	1.298
Espumoso (ESP)	-	-	-
Ivorá (IV)	-11.774**	1.381	0.752
Nova Palma (PA)	-	-	-
Nova Petrópolis (NP)	0.856	-	-
Rolante (ROL)	-	-	-
Salto do Jacuí (SJ)	-	-	-
Santa Maria (SM)	0.800	0.019	0.996
São João do Polênise (POL)	6.931	-0.528	1.471*
São Sepé (SS)	-2.980**	-1.629*	-1.953
Segredo (SG)	2.263	1.079	0.935
Soledade (SOL)	4.903	2.224	1.362
Tapera (TAP)	-	-	-
Vale do Sol (VS)	-2.436*	-0.172	0.149

4 - CONCLUSÕES

Na presente dissertação, constatamos que *A. longirostri* é composta por um complexo de espécies, formando um grupo polifilético. Surpreendentemente, os métodos de delimitação de espécies indicaram a presença de pelo menos 14 potenciais espécies crípticas. Os resultados obtidos são robustos e merecem atenção adicional, sendo que o status de espécie deve ser avaliado adicionando evidências ecológicas e morfológicas. Dada a limitação dos caracteres diagnósticos dos eglídeos, é de extrema importância procurar novos caracteres e/ou técnicas (microscopia eletrônica, morfologia de apêndices reprodutivos) para a identificação e descrição das espécies.

Observamos a presença de dois principais clados (Norte e Sul) que, embora geograficamente próximos, são geneticamente muito distintos. Dentro desses dois principais clados (também não-monofiléticos), diversos subclados (potenciais espécies crípticas) bem suportados foram recuperados. Quando considera-se a árvore filogenética que inclui representantes dos principais clados da classificação de Pérez-Losada et al. (2004), fica claro que os espécimes do Norte e Sul pertencem a clados distintos, sendo estritamente mais relacionadas com diversas outras espécies de eglídeos do que são entre elas.

Possivelmente há uma influência da altitude na diversificação das populações crípticas. As populações do clado Norte encontram-se em uma área de alta altitude com superfície ondulada e são geneticamente distantes entre elas. As populações do clado Sul estão em uma área de baixa altitude com a superfície relativamente plana e suas populações são geneticamente mais próximas, quando comparadas com as populações do Norte. Além disso, os ancestrais dessas populações parecem ter colonizado as regiões por diferentes caminhos e em mais de um evento. A diferença de altitudes entre as regiões pode ter agido como barreira, restringindo a conectividade entre as populações dos diferentes clados.

Sobre a origem das possíveis espécies, a maioria teve origem durante o Pleistoceno Superior (126,000 – 11,700 anos atrás), sendo que entre o fim do Pleistoceno e o início do Holoceno (~40,000 – 8,000 anos atrás) o clima era predominantemente seco (SPALDING et al., 2015). As populações de eglídeos possivelmente ficaram isoladas durante períodos de seca, o que deve ter reduzido ou até impedido o fluxo gênico, o primeiro passo para especiação. Como esses eventos são recentes, a evolução fenotípica pode não ter acompanhado a rápida

divergência encontrada no mtDNA, explicando a diversidade críptica encontrada em *A. longirostri*.

Espera-se que os resultados aqui obtidos possam contribuir para o entendimento sobre a história evolutiva dos eglídeos. Acredito que futuros estudos podem utilizar os dados aqui encontrados para esclarecer as relações entre os principais clados de Pérez-Losada et al. (2004), principalmente os clados C,D e E. Também, com base na possível contribuição do relevo na diversificação dos caranguejos, que futuros trabalhos possam investigar o tema mais a fundo, delineando coletas levando em conta as formações geológicas dos locais. A grande diversidade encontrada no complexo *A. longirostri* pode servir de exemplo e instigar novas pesquisas que busquem elucidar a diversidade críptica no gênero, tema muito importante não só para estimativas mais precisas da biodiversidade, mas também para a sua conservação. Certamente, a real diversidade do gênero ainda é muito subestimada. A busca por novos caracteres diagnósticos ou novas técnicas para a delimitação de espécies em *Aegla* se torna necessária para que as espécies crípticas possam ser formalmente descritas e nomeadas. Por fim, que essas informações possam servir de subsídio para aplicar medidas mais efetivas de gestão e conservação da diversidade biológica.

REFERÊNCIAS BIBLIOGRÁFICAS

ABELL, R. Conservation biology for the biodiversity crisis: a freshwater follow-up. **Conservation Biology.** v. 16, p. 14-37, 2002.

ARENAS, J. La cordillera de la costa como refugio de la fauna dulcícola preglacial. **Archivos de Biología y Medicina Experimentales.** v. 10, p. 1-40, 1974.

AVISE, J. C. Phylogeography: the history and formation of species. **Harvard university press.** 2000.

AVISE, J. C. Phylogeography: retrospect and prospect. **Journal of biogeography.** v. 36, p. 3-15, 2009.

AVISE, J. C.; BOWEN, B. W.; AYALA, F. J. In the light of evolution X: Comparative phylogeography. **Proceedings of the National Academy of Sciences**. v. 113, n. 29, p. 7957-7961, 2016.

BARBER, B. R.; XU, J.; PÉREZ-LOSADA, M.; JARA, C. G.; CRANDALL, K. A. Conflicting evolutionary patterns due to mitochondrial introgression and multilocus phylogeography of the Patagonian freshwater crab *Aegla neuquensis*. **PloS one.** v. 7, p. e37105, 2012.

BARTHOLOMEI-SANTOS, M. L.; RORATTO, P. A.; SANTOS, S. High genetic differentiation of *Aegla longirostri*(Crustacea, Decapoda, Anomura) populations in southern Brazil revealed by multi-loci microsatellite analysis. **Genetic and Molecular Research.** v. 10, p. 4133–4146, 2011.

BICKFORD, D.; LOHMAN, D. J.; SODHI, N. S.; NG, P. K. L.; MEIER, R.; WINKER, K.; INGRAM, K. K.; DAS, I. Cryptic species as a window on diversity and conservation. **Trends in Ecology and Evolution.** v 22, p.148–155, 2007.

BOND-BUCKUP, G.; BUCKUP, L. A família Aeglidae (Crustacea, Decapoda, Anomura)**.** **Arquivos de Zoologia.** v. 32, p. 159–346, 1994

BOND-BUCKUP, G.; JARA, C. G.; PÉREZ-LOSADA, M.; BUCKUP, L; CRANDALL, K. A. Global diversity of crabs (Aeglidae: Anomura: Decapoda) in freshwater. **Hydrobiologia.** v. 595, p. 267–273, 2008.

BOND-BUCKUP, G.; JARA, C. G.;BUCKUP, L.; PÉREZ-LOSADA, M.; BUENO, A. A. P.; CRANDALL, K. A.; SANTOS, S. New species and new records of endemic freshwater crabs from the Atlantic Forest in southern Brazil (Anomura: Aeglidae). **Journal of Crustacean Biology.** v. 30, p. 495–502, 2010.

BRACKEN-GRISSOM, H. D.; CANNON M. E.; CABEZAS, P.; FELDMANN, R. M.; SCHWEITZER, C. E.; AHYONG, S.T.; FELDER, D. L.; LEMAITRE, R.; CRANDALL, K. A. A comprehensive and integrative reconstruction of evolutionary history for Anomura (Crustacea: Decapoda). **BMC Evolutionary Biology.** v.13, p. 128, 2013.

BUENO, A. A. P.; BOND-BUCKUP, G. Natural Diet of *Aegla platensis* Schmitt and *Aegla ligulata* Bond-Buckup & Buckup (Crustacea, Decapoda, Aeglidae) from Brazil. **Acta Limnologica Brasiliensia.** v. 16, p. 115-127, 2004.

CASSINI, M. H.; FASOLA, L.; CHEHÉBAR, C.; MACDONALD, D. W. Scale-dependent analysis of an otter–crustacean system in Argentinean Patagonia. **Naturwissenschaften**. v. 96, p. 593-599, 2009.

CLAPPERTON, C. M. Nature of environmental changes in South America at the Last Glacial Maximum. **Palaeogeography, palaeoclimatology, palaeoecology**. v. 101, p. 189-208, 1993.

COGO, G. B.; SANTOS, S. The role of aeglids in shredding organic matter in neotropical streams. **Journal of Crustacean Biology.** v. 33, p. 519-526, 2013.

COLLINS, P. A.; GIRI, F.; WILLINER, V. Biogeography of the freshwater decapods in the La Plata basin, South America. **Journal of Crustacean Biology**. v. 31, p. 179-191, 2011.

COOK, B.; PAGE, T.; HUGHES, J. Importance of cryptic species for identifying 'representative' units of biodiversity for freshwater conservation. **Biological Conservation.** v. 141, p. 2821-2831, 2008.

CRACRAFT, J. Species concepts and speciation analysis. **Current Ornithology.** v. 1, p. 159–187, 1983.

DA SILVA, J. M.; CREER, S.; DOS SANTOS, A.; COSTA, A. C.; CUNHA, M. R.; COSTA, F. O.; CARVALHO, G. R. Systematic and evolutionary insights derived from mtDNA COI barcode diversity in the Decapoda (Crustacea: Malacostraca). **PLoS one**. v. 6, e19449, 2011.

DANIELS, S. R.; GOUWS, G.; STEWART, B. A.; COKE, M. Molecular and morphometric data demonstrate the presence of cryptic lineages among freshwater crabs (Decapoda: Potamonautidae: Potamonautes) from the Drakensberg Mountains, South Africa. **Biological Journal of the Linnean Society**. v. 78, p. 129-147, 2003.

DE CARVALHO, F. L.; PILEGGI, L. G.; MANTELATTO, F. L. Molecular data raise the possibility of cryptic species in the Brazilian endemic prawn *Macrobrachium potiuna* (Decapoda, Palaemonidae). **Latin American Journal of Aquatic Research**. v. 41, p. 707, 2013.

FELDMANN, R. M. Haumuriaegla glaessneri n. gen. and sp. (Decapoda; Anomura; Aeglidae) from Haumurian (Late Cretaceous) rocks near Cheviot, New Zealand. **New Zealand journal of geology and geophysics.** v. 27, p. 379–385, 1984.

FELDMANN, R. M.; VEGA, F. J.; APPLEGATE, S. P.; G. A. BISHOP, G. A. Early Cretaceous arthropods from the Tlayúa formation at Tepexi de Rodríguez, Puebla, México. **Journal of Paleontology.** v. 72, p. 79–90, 1998.

FRANKHAM R.; BALLOU, J. D.; BRISCOE, D. A. A Primer of Conservation Genetics. **Cambridge University Press. New York**, 2004.

HEPP L. U.; FORNEL, R.; RESTELLO, R. M.; TREVISAN, A.; SANTOS S**.** Intraspecific morphological variation in a freshwater crustacean *Aegla plana* in Southern Brazil: effects of geographical isolation on carapace shape. **Journal of Crustacean Biology.** v. 32, p. 511-518, 2012.

HURST, G. D.; JIGGINS, F. M. Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. **Proceedings of the Royal Society B: Biological Sciences**. v. 272, p. 1525-1534, 2005.

JARA C. G.; PEREZ-LOSADA, M.; CRANDALL, K. A. A new species of freshwater anomuran crab of the genus *Aegla* Leach, 1821 (Crustacea: Decapoda: Aeglidae) from the Nahuelbuta Coastal Range, Chile. **Proceedings of the Biological Society of Washington.** v. 116, p. 933-942, 2003.

LARA, G.; MORENO, C. Efectos de la depredación de *Aegla abtao* (Crustacea, Aeglidae) sobre la distribución espacial y abundancia de Diplodon chilensis (Bivalvia, Hyriidae) en el Lago Panguipulli, Chile. **Revista Chilena de Historia Natural.** v. 68, p. 123-129, 1995.

LUNDBERG, J. G. The temporal context for the diversification of Neotropical fishes. **Phylogeny and classification of Neotropical fishes**. p. 49-68, 1998.

MACHORDOM, A.; MACPHERSON, E. Rapid radiation and cryptic speciation in squat lobsters of the genus *Munida* (Crustacea, Decapoda) and related genera in the South West Pacific: molecular and morphological evidence. **Molecular phylogenetics and evolution**. v. 33, p. 259-279, 2004.

MAGNI, S. T.; PY-DANIEL, V. *Aegla platensis* Schmitt, 1942 (Decapoda: Anomura) a predator of Simuliidae imature stages (Diptera: Culicomorpha). **Revista de Saúde Pública**. v. 23, p. 258-259, 1989.

MANTELATTO, F. L.; PEZZUTO, P. R.; MASELLO, A.; WONGTSCHOWSKI, C. L. D.; HILSDORF, A. W. S.; ROSSI, N. Molecular analysis of the commercial deep-sea crabs *Chaceon ramosae* and Chaceon notialis (Brachyura, Geryonidae) reveals possible cryptic species in the South Atlantic. **Deep Sea Research Part I: Oceanographic Research Papers.** v. 84, p. 29-37 2013.

MARCHIORI, A. B.; BARTHOLOMEI-SANTOS, M. L.; SANTOS, S. Intraspecific variation in *Aegla longirostri* (Crustacea: Decapoda: Anomura) revealed by geometric morphometrics: evidence for ongoing speciation? **Biological Journal of the Linnean Society.** v. 112, p. 31-39, 2014.

MELO, M. T. Q. **Dieta de *Caiman latirostris* (Daudin, 1802) na Estação Ecológica do Taim, RS.** Dissertação. Pontifícia Universidade Católica do Rio Grande do Sul, Instituto de Biociências, Porto Alegre. p. 63, 1990.

MORAES, J. C. B.; TEROSSI, M.; BURANELLI, R. C.; TAVARES, M.; MANTELATTO, F. L.; BUENO, S. L. D. S. (2016). Morphological and molecular data reveal the cryptic diversity among populations of *Aegla paulensis* (Decapoda, Anomura, Aeglidae), with descriptions of four new species and comments on dispersal routes and conservation status. **Zootaxa.** v. 4193, p. 1-48, 2016.

ORTMANN, Arnold Edward. The geographical distribution of freshwater decapods and its bearing upon ancient geography. **Proceedings of the American Philosophical Society**. v. 41, p. 267-400, 1902.

PÉREZ-LOSADA, M.; BOND-BUCKUP, G.; JARA, C. G.; CRANDALL, K. A. Molecular systematics and biogeography of the southern South american freshwater "crabs" *Aegla* (Decapoda: Anomura: Aeglidae) using multiple heuristic tree search approaches. **Systematic Biology.** v. 53, p. 767-780, 2004.

PÉREZ-LOSADA, M.; BOND-BUCKUP, G.; JARA, C. G.; CRANDALL, K. A. Conservation assessment of Southern South American freshwater ecoregions on the basis of the distribution and genetic diversity of crabs from the genus *Aegla*. **Conservation Biology.** v. 23, p. 692-702, 2009.

PROVAN, J.; BENNETT, K. D. Phylogeographic insights into cryptic glacial refugia. **Trends in ecology & evolution**. v. 23, p. 564-571, 2008.

RIDDLE, B. R.; HAFNER, D. J. A step-wise approach to integrating phylogeographic and phylogenetic biogeographic perspectives on the history of a core North American warm deserts biota. **Journal of Arid Environments.** v. 66, p. 435-461, 2006.

RINGUELET, R. A. Consideraciones sobre las relaciones filogenéticas entre las especies del género *Aegla* Leach (Decápodos, Anomuros). **Notas Universidad Nacional de La Plata, Instituto del Museo.** v. 14, p. 111–118, 1949.

SANTOS, S.; AYRES-PERES, L.; CARDOSO, R. C.; Sokolowicz, C. C. Natural diet of the freshwater anomuran *Aegla longirostri* (Crustacea, Anomura, Aeglidae). **Journal of Natural History**. v. 42, p. 1027-1037, 2008.

SANTOS, S.; JARA, C. G.; BARTHOLOMEI- SANTOS, M. L.; PÉREZ-LOSADA, M.; CRANDALL, K. A. New species and records of the genus *Aegla* Leach, 1820 (Crustacea, Anomura, Aeglidae) from the West-Central region of Rio Grande do Sul, Brazil. **Nauplius**, v. 21, p. 211-223, 2013.

SANTOS, S.; BOND-BUCKUP, G.; BUCKUP, L.; BARTHOLOMEI-SANTOS, M. L.; PÉREZ-LOSADA, M.; JARA, C. G.; CRANDALL, K. A. Three new species of Aeglidae (*Aegla* Leach, 1820) from Paraná State, Brazil. **Journal of Crustacean Biology.** v. 35, p. 839-849, 2015.

SANTOS, S.; BOND-BUCKUP, G.; GONÇALVES, A. S.; BARTHOLOMEI-SANTOS, M. L.; BUCKUP L, JARA, C. G. Diversity and conservation status of *Aegla* spp. (Anomura, Aeglidae): an update. **Nauplius.** X:000-000, 2016.

SCHMITT, W. The species of *Aegla* endemic South American freshwater crustaceans. **Proceedings of the United States National Museum**. v. 91, p. 431–520, 1942.

SPALDING B. B. C.; LORSCHEITTER, M. L. Dry and humid phases in the highlands of southern Brazil during the last 34,000 years, and their influence on the paleoenvironments of the region. **Quaternary International.** v. 377, p. 102-111, 2015

XU, J.; PÉREZ-LOSADA, M.; JARA, C. G.; CRANDALL, K. A. Pleistocene glaciation leaves deep signature on the freshwater crab *Aegla alacalufi* in Chilean Patagonia. **Molecular Ecology**. v. 18, p. 904-918, 2009.

ZIMMERMANN, B. L.; DAMBROS, C. S.; SANTOS, S. Association of microhabitat variables with the abundance and distribution of two neotropical freshwater decapods (Anomura: Brachyura). **Journal of Crustacean Biology**. v. 36, p. 198-204, 2016.

ZINK, R. M.; BARROWCLOUGH, G. F. Mitochondrial DNA under siege in avian phylogeography. **Molecular Ecology.** v. 17, p. 2107-2121, 2008.