UNIVERSIDADE FEDERAL DE LAVRAS

STELLA GOMES RODRIGUES

FILOGENIA MOLECULAR, BIOGEOGRAFIA E ESTRUTURA POPULACIONAL DE ANFÍPODOS DE ÁGUA DOCE (CRUSTACEA, HYALELLIDAE) DA AMÉRICA DO SUL

LAVRAS - MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ecologia Aplicada, área de concentração em Ecologia e Conservação de Recursos em Paisagens Fragmentadas e Agrossistemas, para a obtenção do título de Doutor.

Orientadora

Dra. Alessandra Angélica de Pádua Bueno

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Dr. Fernando Luis Medina Mantelatto	USP-RP
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“Nada em Biologia faz sentido exceto à luz da Evolução.”

Theodosius Dobzhansky

RESUMO

Os ecossistemas dulcícolas são considerados atualmente os ambientes mais ameaçados do planeta. Diversas espécies de água doce estão sendo extintas antes mesmo de serem descritas e a situação é ainda mais grave para os invertebrados, como os crustáceos dulcícolas do gênero *Hyalella*. A existência de espécies crípticas em *Hyalella* dificulta o desenvolvimento de estudos sobre esses animais, bem como na criação de políticas de conservação direcionadas a eles. Estudar a ecologia e filogenia molecular de *Hyalella* irá auxiliar no conhecimento da sua diversidade e distribuição geográfica, no delineamento de suas espécies, nas relações evolutivas dentro do gênero e na estimativa de sua efetiva situação de ameaça. Assim, essa tese foi realizada com o objetivo geral de se conhecer a filogenia molecular, a biogeografia e alguns aspectos populacionais de *Hyalella* na América do Sul. O primeiro capítulo apresenta uma revisão bibliográfica sobre o tema estudado, bem como os objetivos e hipóteses da autora. O segundo capítulo estudou a filogenia molecular do gênero e sua história biogeográfica na América do Sul. Para isso, um gene nuclear (H3) e dois mitocondriais (16S e 12S) foram parcialmente sequenciados e as relações das espécies foram elucidadas através de análises de Máxima Verossimilhança e Inferência Bayesiana. Um impressionante número de 20 novas espécies foi encontrado, bem como a existência de um complexo de espécies. Os subgêneros de *Hyalella* não formaram um clado monofilético e o gênero possui uma provável origem no Eoceno, com um pico de diversificação no Mioceno. O terceiro capítulo da tese estudou aspectos populacionais de quatro espécies de *Hyalella* no Brasil, oriundas de diferentes latitudes e biomas. As populações foram coletadas em duas estações diferentes do ano de 2012. Em geral, a reprodução das espécies próximas aos trópicos foi contínua ao longo do ano e das espécies de altas latitudes a reprodução foi sazonal. O tamanho corporal dos indivíduos de latitudes altas é maior do que dos indivíduos das espécies de latitudes mais baixas. Além disso, a maturidade sexual das espécies próximas dos trópicos é alcançada em tamanhos menores. A fecundidade das espécies aparentemente está mais relacionada com o tamanho corporal da fêmea do que com o clima e latitude. Essa tese é o primeiro trabalho que utiliza técnicas moleculares a fim de se conhecer as relações evolutivas de *Hyalella* na América do Sul e realiza a comparação de aspectos populacionais de espécies geograficamente distantes. A filogenia molecular e as informações ecológicas do gênero obtidas nessa tese auxiliarão na conservação de ecossistemas de água doce na América do Sul, visto que esses organismos são endêmicos e bioindicadores de qualidade ambiental.

Palavras-chave: Crustáceos, ecologia, *Hyalella*, filogenia molecular, Peracarida.

ABSTRACT

Freshwater ecosystems are considered the most threatened environments on the planet. Several freshwater species are becoming extinct before being described and the situation is even worse for invertebrates, such as the amphipod crustaceans *Hyalella*. The existence of cryptic species of *Hyalella* difficult the development of studies about these animals, as well as the creation of conservation policies addressed to them. Studying the ecology and the molecular phylogeny of *Hyalella* will aid to understand its diversity and geographic distribution, in the delimitation of its species, in the evolutionary relationships within the genus and on the estimate of their actual threat situation. Thus, this thesis was performed with the aim of know the molecular phylogeny, the biogeography and some population aspects of *Hyalella* in South America. The first chapter presents a literature revision about the topic studied, as well as the aims and hypothesis of the author. The second chapter studied the molecular phylogeny of the genus and its biogeographic history in South America. For this, one nuclear gene (H3) and two mitochondrial (16S and 12S) were partially sequenced and the relationships of the species were elucidated by Maximum Likelihood and Bayesian Inference analyzes. An impressive number of 20 new species was found, as well as the existence of a species complex. The subgenus of *Hyalella* did not form a monophyletic group and the genus has a origin probable in Eocene, with a peak of diversification in Miocene. The third chapter presents the study of the population aspects of four species of *Hyalella* in Brazil, from different latitudes and biomes. Populations were sampled in two seasons of 2012. In general, the reproduction of the species closer to the tropics was continuous throughout the year and in species from high latitudes the reproduction was seasonal. Body size of individuals in high latitudes is larger than individuals of lower latitudes. Moreover, sexual maturity of the species closer to tropics is reached at smaller body sizes. The fecundity of the species is apparently more related to the female's body size than to the climate and latitude. This thesis is the first study that uses molecular techniques in order to understand the evolutionary relationships of *Hyalella* in South America and performs the comparison of population aspects of geographically distant species. Molecular phylogeny and ecological information of the genus obtained from this thesis will aid in the conservation of freshwater ecosystems in South America, as these organisms are endemic and bioindicators of water quality.

Keywords: Crustaceans. Ecology. *Hyalella*. Molecular phylogeny. Peracarida.

SUMÁRIO

PRIMEIRA PARTE

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1 INTRODUÇÃO GERAL

Os ecossistemas dulcícolas são considerados atualmente os ambientes mais ameaçados do planeta. A perda de biodiversidade na água doce é muito superior a dos ambientes marinhos e terrestres, e diversas espécies estão sendo extintas antes mesmo de serem descritas pela ciência. A situação é mais grave para os invertebrados, pois sequer existem estimativas sobre sua situação de ameaça. Grupos de invertebrados que vivem exclusivamente em ambiente aquático, como a maioria dos crustáceos, estão entre os organismos mais vulneráveis à impactos ambientais, pois facilmente podem ser extintos.

Os crustáceos anfípodos de água doce do gênero *Hyalella* Smith, 1874 se enquadram nessa situação. Suas espécies são sensíveis a impactos ambientais, são utilizadas em testes de toxicidade e apresentam populações endêmicas com baixa capacidade de dispersão, o que as torna muito susceptíveis à extinção por fatores antrópicos. *Hyalella* ocorre apenas nas Américas e é muito pouco conhecido na América do Sul, tanto em sua ecologia quanto à sua real diversidade. Além disso, *Hyalella* possui espécies de morfologia bastante semelhante, o que dificulta a identificação das populações, a descrição de novas espécies e o conhecimento de sua distribuição geográfica. Conhecer a ecologia e filogenia molecular desses organismos são ferramentas que irão auxiliar no conhecimento da diversidade, no delineamento de espécies e sua distribuição, nas relações de parentesco do gênero e em sua efetiva situação de ameaça.

Dessa forma, essa tese foi realizada com o objetivo geral de se conhecer a filogenia molecular, a biogeografia e os aspectos das populações de diferentes espécies de *Hyalella* da América do Sul. Assim, esse trabalho está dividido em três capítulos. O primeiro fornece informações sobre o tema estudado na tese, onde foi realizada uma ampla revisão bibliográfica sobre a atual situação dos ecossistemas aquáticos dulcícolas no mundo e no Brasil, destacando a importância de incluí-los em estudos de ecologia e conservação. Além disso, o referencial teórico aborda os crustáceos anfípodos quanto à sua diversidade, distribuição, história taxonômica, e as pesquisas mais atuais sobre sua ecologia e filogenia molecular. Ademais, esse capítulo destaca a importância de se conhecer a dinâmica populacional e a filogenia molecular de um grupo, com propósitos de conservação ambiental. Por fim, no final do primeiro capítulo estão incluídos os objetivos e as hipóteses da autora ao realizar a tese.

O segundo capítulo foi redigido na forma de artigo e trata da primeira filogenia molecular realizada para as espécies de *Hyalella* da América do Sul. Além disso, é discutido se as espécies brasileiras do gênero são monofiléticas e se os subgêneros de *Hyalella* são válidos, além de ser estimado o tempo de diversificação do grupo e sua história biogeográfica na América do Sul. Por fim, o terceiro capítulo, que também foi escrito na forma de artigo, apresenta uma pesquisa realizada com quatro espécies de *Hyalella* no Brasil, em diferentes biomas e latitudes. Foram analisados diversos aspectos das populações, como período reprodutivo, tamanho corporal, maturidade e razão sexual, fecundidade, entre outros.

Essa tese é pioneira pois utiliza técnicas moleculares na tentativa de se conhecer as relações evolutivas das espécies de *Hyalella* da América do Sul, especialmente do Brasil. A estimativa da filogenia aliada a informações ecológicas das espécies poderão auxiliar na criação de programas de conservação de ecossistemas de água doce no Brasil, visto que esses organismos são extremamente endêmicos e podem ser bioindicadores de qualidade ambiental. Esse trabalho será referência para futuras pesquisas com *Hyalella* e outros anfípodos, de forma que com esses resultados será possível construir a filogenia de todo o gênero, incluindo as espécies que ocorrem na América Central e do Norte. Ademais, poderá ser estimado de forma mais precisa quando ocorreu a ocupação de *Hyalella* pelo continente.

2 REFERENCIAL TEÓRICO

2.1 Os ecossistemas aquáticos dulcícolas

Biodiversidade pode ser definida como a variedade de ecossistemas, espécies e suas diferentes populações, bem como a diversidade genética das mesmas. A diversidade biológica do planeta está rapidamente sendo esgotada como consequência direta e indireta das ações humanas (FRANKHAM; BALLOU; BRISCOE, 2002). Um número desconhecido de espécies já foram extintas e muitas tiveram suas populações reduzidas a tamanhos tão pequenos que estão em perigo de desaparecer. O problema atual é tão grande que já está sendo chamado de "a sexta grande extinção", pois sua magnitude se compara com as outras cinco grandes extinções em massa que ocorreram na Terra (FRANKHAM; BALLOU; BRISCOE, 2002). Dessa forma, a biologia da conservação é motivada pela necessidade de reduzir as atuais taxas de extinção e de preservar a biodiversidade (FRANKHAM; BALLOU; BRISCOE, 2002; EGUIARTE; SOUZA; AGUIRRE, 2007).

Apesar de fazerem parte de toda e qualquer atividade humana, os ecossistemas de água doce contém apenas 0,01% de toda a água do planeta e cobrem somente 0,8% de sua superfície (ABELL, 2002). Atualmente, os ambientes dulcícolas são considerados os ecossistemas mais ameaçados do mundo, de forma que os principais perigos são: alteração do habitat e degradação (eutrofização, acidificação, sedimentação, aumento da turbidez, remoção da vegetação ripária, canalização); contaminação por substâncias tóxicas; introdução de espécies exóticas; manipulações hidrológicas (reservatórios, remoção de águas subterrâneas, uso na irrigação); sobre-exploração de espécies com importância econômica e pressões globais (aumento da incidência de luz ultravioleta, aquecimento global) (DUDGEON et al., 2006;

SUSKI; COOKE, 2006). Esse cenário é ainda pior para os habitats dulcícolas de tamanho reduzido, como lagos e nascentes, bem como para as espécies endêmicas que ocorrem nesses ambientes. Dessa forma, acredita-se que a perda da biodiversidade na água doce exceda muito a dos ambientes marinhos e terrestres (NEL et al., 2009).

Cerca de um terço de todas as espécies de vertebrados do planeta estão confinadas à água doce. Os peixes dulcícolas, por exemplo, compreendem 25% de todas as espécies atuais de vertebrados, e estima-se que dessas cerca de 30% estão ameaçadas. Contudo, a situação é ainda mais grave para os invertebrados, pois apesar de compreenderem grande parte da biodiversidade desses ecossistemas, não existem estimativas sobre a sua real situação de ameaça (ABELL, 2002).

O conhecimento da diversidade dos invertebrados dulcícolas é precário, sendo particularmente maior nos trópicos, regiões que concentram grande parte das espécies da Terra (ABELL, 2002). Além disso, os invertebrados aquáticos possuem um papel essencial nas cadeias tróficas, pois além de ocuparem diferentes níveis (atuando como predadores, herbívoros, detritívoros, decompositores, servindo de alimento para invertebrados e vertebrados, entre outros), muitos deles completam seu ciclo de vida em terra, sendo importantes tanto para os ambientes de água doce como para os terrestres (DUDGEON et al., 2006).

Assim, devido às pressões antrópicas que sofrem, do contínuo e desenfreado uso para a sobrevivência humana, da necessidade urgente de criação de medidas de conservação e da carência de conhecimento da diversidade, são urgentes os trabalhos que estudem os ecossistemas dulcícolas, principalmente os que ocorrem em latitudes tropicais. Além disso, qualquer estudo que aborde a ecologia, biologia, taxonomia, genética e outros aspectos dos invertebrados aquáticos de água doce, irá contribuir substancialmente com a conservação dos ecossistemas dulcícolas, bem como de toda sua biodiversidade.

2.2 Filogenia molecular

De acordo com a teoria da evolução proposta por Darwin, todos os organismos evoluíram de um único ancestral comum, que seria a origem da vida. Dessa forma, diferentes mecanismos genéticos levaram ao surgimento da biodiversidade encontrada hoje, como as mutações, duplicação de genes, reorganização de genomas e trocas genéticas como a recombinação, rearranjo e transferência horizontal de genes (LEMEY; SALEMI; VANDAMME, 2009).

Levando-se em conta essa informação, é possível investigar a evolução e as relações entre os organismos, espécies e até genes, utilizando diferentes tipos de dados. A maneira clássica de se estimar as relações entre as espécies é comparar seus caracteres morfológicos, sendo esse o principal método em que a taxonomia se baseia (FRANKHAM, 1995). Contudo, nas últimas décadas, houve um exponencial aumento das informações moleculares disponíveis, como sequências de aminoácidos e nucleotídeos, por exemplo, que também podem ser utilizadas para inferir sobre relações filogenéticas (CRANDALL; FITZPATRICK, 1996).

Nos últimos anos, muitos foram os avanços metodológicos em biologia molecular. A obtenção de dados moleculares tornou-se uma importante ferramenta para o entendimento de complexas relações filogenéticas, onde apenas o uso de caracteres morfológicos não é suficiente. Além disso, essas metodologias tem a vantagem de utilizar caracteres universais e conservativos, além de poderem ser utilizadas no delineamento efetivo dos limites entre linhagens e/ou espécies (AGAPOW et al., 2004; EGUIARTE; SOUZA; AGUIRRE, 2007). As análises filogenéticas moleculares também estabelecem as relações entre genes ou seus fragmentos, através da inferência de sua história comum. Basicamente, métodos filogenéticos consideram a similaridade entre os genes, assumindo que são homólogos, ou seja, compartilham de um mesmo ancestral comum (EGUIARTE; SOUZA; AGUIRRE, 2007).

Apesar de ser um consenso que todos os organismos compartilham de um mesmo ancestral, ao longo do tempo a similaridade entre dois genes pode ser perdida de tal forma que as próprias sequências de dados não carregam informação suficiente sobre a relação entre dois genes, já que esses acumularam muita variação. Além disso, o termo homologia é usado somente quando o ancestral comum é recente o suficiente para que as informações das sequências tenham retido similaridade suficiente para que seja usada nas análises filogenéticas (LEMEY; SALEMI; VANDAMME, 2009).

Algumas espécies de animais são particularmente promissoras no campo da genética da conservação. Táxons endêmicos, como famílias e gêneros de crustáceos de água doce, incluindo lagostins e anfípodos, são organismos potenciais no estudo da filogenia molecular, filogeografia, biogeografia e conservação de ecossistemas (CRANDALL; FITZPATRICK, 1996; CRANDALL et al., 2000a; CRANDALL et al., 2000b). Esses animais possuem características essenciais para estudos de conservação, como a distribuição restrita a algumas áreas, ou até mesmo continentes, e genes com áreas relativamente conservativas, facilitando as estimativas de filogenia (ENGLISCH et al., 2003).

As principais questões genéticas da biologia da conservação incluem a ocorrência de endogamia, acumulação e perda por mutações deletérias, perda de variação genética em populações pequenas, fragmentação de populações, redução na migração e incertezas taxonômicas. Em decorrência disso, análises moleculares que estudem espécies-chave, como os citados crustáceos de água doce, são extremamente relevantes para o manejo e conservação desses organismos e dos ambientes em que vivem (DE QUEIROZ; GAUTHIER, 1992; FRANKHAM, 1995; EGUIARTE; SOUZA; AGUIRRE, 2007).

Dessa forma, atualmente, a filogenia molecular é uma das principais ferramentas da biologia da conservação, e deve ser usada no estudo de espécies ameaçadas, na criação de programas de conservação e preservação de todos os tipos de ecossistemas, bem como no entendimento da distribuição de organismos e conhecimento de sua biogeografia. Aliada a outras ferramentas, como a taxonomia e estudos ecológicos aplicados, a filogenia molecular certamente está entre as metodologias mais promissoras para auxiliar na conservação da biodiversidade (EGUIARTE; SOUZA; AGUIRRE, 2007).

2.3 Dinâmica populacional

A conservação de todo ecossistema depende do conhecimento dos aspectos ecológicos das populações que o compõe. Compreender a abundância e o tamanho de uma população, sua estabilidade, produtividade, função na cadeia trófica, taxas de imigração e emigração, período reprodutivo, recrutamento de jovens, tempo médio de vida, entre tantos outros aspectos, são cruciais para que ocorra uma conservação a longo prazo (COOPER, 1965; HUTCHINSON, 1981; MUSKÓ, 1993). Além dessas características que são inerentes de cada população, seus indivíduos também interagem entre si, com o ambiente e outras populações, seja na busca por um parceiro reprodutivo, na competição por alimento, espaço e outros recursos (FONTELES-FILHO, 1989).

Todas as informações sobre a dinâmica populacional de uma espécie embasam e norteiam a criação de programas de conservação, a avaliação de seu risco de extinção e de um possível uso sustentável, bem como de seu potencial para uso em estudos de bioindicação e ecotoxicologia (BRAWN; ROBINSON, 1996).

Populações de animais de água doce que não possuem um estágio de vida que seja dispersor, geralmente são pequenas, isoladas e endêmicas, como os crustáceos da ordem Amphipoda (BARNARD; BARNARD, 1982). Assim, conhecer a dinâmica populacional desses organismos é de vital importância para verificar a existência de um declínio no crescimento da população ou até mesmo de endogamia, fatores que podem levá-la à extinção (SACCHERI; HANSKI, 2006).

Os crustáceos anfípodos de água doce estão entre os organismos mais comumente utilizados em avaliações de qualidade ambiental, como *Hyalella azteca* (Saussure, 1858) e *Gammarus lacustri* Sars, 1863, espécies que ocorrem somente no Hemisfério Norte. Esses crustáceos são considerados bioindicadores de boa qualidade de água devido à sua alta sensibilidade quando na presença de contaminantes e outros impactos ambientais (NEUPARTH; COSTA; COSTA, 2002; WILCOXEN; MEIER; LANDRUM, 2003; DING et al., 2011).

Protocolos de avaliação de qualidade ambiental criados a partir de estudos com espécies de clima temperado não podem ser usados no Brasil. Além disso, a biologia e a ecologia das espécies norte-americanas são extremamente diferentes dos organismos nativos do Brasil, já que eles são adaptados a outro tipo de hábitat, possuem outra fisiologia, ciclo de vida, período de reprodução, etc. (PHILLIPS; SEGAR, 1986; McELARNEY; RIPPEY, 2009).

Dessa forma, estudar a dinâmica populacional das espécies nativas de anfípodos de água doce do Brasil se torna essencial para que se possa criar programas de conservação dos ecossistemas aquáticos do país, além de protocolos de avaliação de qualidade ambiental adequados para os hábitats brasileiros.

2.4 Os crustáceos anfípodos de água doce

Os crustáceos possuem a maior variedade morfológica dentre todos os grupos animais existentes e conquistaram praticamente todos os habitats da Terra. Essas características sugerem que esses organismos desenvolveram estratégias sexuais, sociais e de histórias de vida únicas, culminando em um enorme sucesso ecológico e evolutivo (MARTIN; DAVIS, 2001; DUFFY; THIEL, 2007).

Amphipoda é uma das ordens de crustáceos mais bem-sucedidas. Esses invertebrados podem ser encontrados em todo o planeta e sua diversidade é majoritariamente marinha. Entretanto, muitas espécies ocorrem em diferentes habitats dulcícolas e são especialmente abundantes em águas correntes frias e subterrâneas de regiões temperadas. Das cerca de 9.100 espécies da ordem, 20% ocorrem em ambientes de água doce e algumas poucas são terrestres (VÄINÖLÄ et al., 2008).

Geralmente, os anfípodos possuem o corpo comprimido lateralmente e apresentam tamanhos que variam de 2 a 40 mm, mais comumente entre 5 e 15 mm. As fêmeas possuem um marsúpio - localizado no pereon - estrutura onde ocorre a fertilização e incubação dos ovos. Diferentemente da maioria dos crustáceos, os anfípodos possuem o desenvolvimento direto, ou seja, os juvenis eclodem dos ovos com a morfologia dos adultos, mas sem as características sexuais secundárias desenvolvidas (BOROWSKY, 1991; MORRIT; SPICER, 1996).

É difícil estimar a idade de surgimento de Amphipoda e de que forma se deu sua ocupação pelo planeta, uma vez que fósseis do grupo são raros. O exoesqueleto dos anfípodos não são tão esclerotizados como em outros crustáceos, o que dificulta a formação de fósseis, de estudos paleontológicos e biogeográficos (KARAMAN, 1984; COLEMAN, 2004). Anfípodos preservados em âmbar não datam antes do Eoceno, contudo, com base em padrões biogeográficos atuais, é estimado que a ordem tenha surgido a pelo menos 250 milhões de anos, e que tenha se diversificado na água doce por volta de 180 milhões de anos atrás, na época de separação da Pangeia (BARNARD; BARNARD, 1982; COLEMAN, 2006; VÄINÖLÄ et al., 2008).

A ausência de um estágio larval em Amphipoda limita a capacidade de dispersão desses animais por longas distâncias, contribuindo para o isolamento geográfico de muitas populações. Dessa forma, as espécies de água doce, principalmente as que ocorrem em lagos e ambientes subterrâneos, muitas vezes são endêmicas ou possuem uma distribuição geográfica limitada (BARNARD; BARNARD, 1982). Porém, já foi observado que aves e mamíferos aquáticos podem auxiliar na dispersão desses crustáceos, pois podem carregá-los em suas penas ou pelos, ajudando no estabelecimento de novas populações (SWANSON, 1984).

Devido aos hábitos primariamente herbívoros e detritívoros, os anfípodos dulcícolas possuem uma grande relevância ecológica. Eles proporcionam a transferência de energia entre os níveis tróficos, atuam na conversão de detritos em matéria orgânica, servem de alimento para aves aquáticas e peixes, e muitas vezes são hospedeiros intermediários de parasitas de vertebrados (WEN, 1992; MUSKÓ, 1993; WELLBORN, 2002; WELLBORN; COTHRAN, 2007).

A sistemática de Amphipoda nos níveis mais altos é extremamente confusa e até o momento nenhuma hipótese filogenética molecular foi realizada. Porém, pelo menos para os táxons dulcícolas, é possível discutir relações evolutivas em níveis de Superfamília, de forma que cada uma possui uma história biogeográfica e de colonização da água doce distintas (LOWRY; MYERS, 2013). Apesar de não existirem estudos filogenéticos abrangentes, os anfípodos de água doce são possivelmente polifiléticos. Sua evolução provavelmente se deu com repetidas colonizações das águas continentais em diferentes épocas e regiões (BARNARD; BARNARD, 1982).

A diversidade de anfípodos de água doce na América do Sul é extremamente baixa, quando comparada com outras regiões do planeta, contando com apenas 10 famílias, 22 gêneros e 74 espécies (FIŠER; ZAGMAJSTER; FERREIRA, 2013). No entanto, essa diversidade certamente é muito maior, principalmente com relação às espécies hipógeas e dentro da família Hyalellidae Bulycheva, 1957 e de seu único gênero, *Hyalella* Smith, 1874. Apenas no Lago Titicaca, por exemplo, que possui uma radiação enorme do gênero, existem provavelmente mais de 100 espécies endêmicas não descritas (GONZÁLEZ; WATLING, 2003).

A razão da diversidade desse continente ser tão baixa é uma questão discutida há pelo menos 30 anos, e duas hipóteses têm sido propostas para explicar o fenômeno (BARNARD; KARAMAN, 1983). A primeira sugere que as linhagens de anfípodos nunca se dispersaram pela América do Sul e a segunda afirma que o recentemente diversificado gênero *Hyalella* (único grupo epígeo de anfípodos de água doce do continente) tenha superado e excluído a fauna ancestral das águas superficiais (BARNARD; KARAMAN, 1983). No trabalho recente de Fišer, Zagmajster e Ferreira (2013), os autores acreditam que todos os táxons de anfípodos estiveram presentes na América do Sul antes da separação de Gondwana, e que *Hyalella* invadiu a água doce apenas quando o continente americano já estava completamente separado das demais massas de terra.

Além disso, os ancestrais dos atuais anfípodos na América do Sul podem ter se tornado extintos nos ambientes superficiais antes do surgimento de *Hyalella*. Esse gênero, por sua vez, deve ter colonizado a superfície com sucesso, resultando em uma diversidade incrível de espécies, que certamente ainda está por ser descrita. Porém, para que ocorra a comprovação dessa hipótese, trabalhos que utilizem a filogenia molecular precisam ser realizados (FIŠER; ZAGMAJSTER; FERREIRA, 2013).

2.5 O gênero *Hyalella*

A família Hyalellidae ocorre somente nas regiões Neártica e Neotropical, desde o sul da Patagônia até a região central do Canadá e apenas o gênero *Hyalella* a compõe. Na América do Sul, *Hyalella* é o único gênero epígeo de anfípodos de água doce, o que o torna extremamente importante nos ecossistemas onde ocorre. Todos os demais táxons de anfípodos da América do Sul estão restritos aos ambientes subterrâneos, como cavernas e habitats hipotelminorreicos (RODRIGUES; BUENO; FERREIRA, 2012; BUENO; RODRIGUES; ARAUJO, 2014).

O gênero é constituído por espécies morfologicamente semelhantes, cuja identificação se torna dificultada pelas descrições antigas e pobres de detalhes, com ilustrações incompletas ou ausentes, em sua maior parte realizadas por pesquisadores não especialistas no grupo. Somando-se a esse quadro, muitos holótipos não foram depositados em coleções científicas ou se perderam, inviabilizando novas análises (BUENO; RODRIGUES; ARAUJO, 2014).

Ao todo, existem 68 espécies de *Hyalella* descritas e a América do Sul é a região que apresenta a maior diversidade, com 52 espécies no total (CARDOSO et al., 2014; RODRIGUES; BUENO; FERREIRA, 2014; COLLA; CÉSAR, 2015; SOUCEK et al., 2015). O Lago Titicaca, localizado entre o Peru e a Bolívia, é a região que concentra o maior número de espécies de *Hyalella* vivendo de forma simpátrica, 14 no total. González e Watling (2003a) afirmam que existem cerca de 100 espécies ainda não descritas para o Titicaca, todas depositadas em Museus. Certamente a riqueza de espécies de *Hyalella* nos países da América do Sul ainda é pouco conhecida e provavelmente subestimada, pois praticamente não existem coletas nos países andinos e em diversas regiões do Brasil.

Os hyalellídeos ocorrem em praticamente todos os tipos de habitats dulcícolas: rios, córregos, lagos, áreas úmidas, nascentes e diversos ambientes subterrâneos. *Hyalella* pode ser dividido em vários grupos de acordo com a sua distribuição geográfica e ocorrência em bacias hidrográficas (GROSSO; PERALTA, 1999; BUENO; RODRIGUES; ARAUJO, 2014).

Bousfield (1996), ao realizar uma revisão sistemática, propôs a subdivisão de *Hyalella* em três subgêneros, baseado somente na morfologia das espécies e em sua distribuição geográfica: *Austrohyalella* (espécies dos Andes e regiões no sul da América do Sul, com características morfológicas mais basais), *Mesohyalella* (espécies do noroeste e leste da América do Sul, incluindo o Brasil, com características morfológicas intermediárias entre os demais subgêneros) e *Hyalella*, sendo esse o subgênero nominal (espécies da América do Norte e América Central, com características morfológicas mais derivadas). Contudo, essa divisão é confusa e nunca foi confirmada ou testada. Além disso, diversas novas espécies foram descritas desde então, muitas delas crípticas, o que dificulta sua identificação e poderia invalidar a alocação das espécies nos subgêneros utilizando apenas sua distribuição geográfica.

Pouco se sabe sobre a idade e origem de *Hyalella,* bem como ocorreu sua invasão na água doce e distribuição pelas Américas. Possivelmente seu ancestral tenha sido um anfípodo marinho que ocorria no Mar de Tétis, durante a Era Mesozoica. O Mar de Tétis teria surgido quando se iniciou a ruptura da Pangeia, formando as massas continentais da Laurásia e Gondwana (GONZÁLEZ, 2001).

Uma hipótese sugere, então, que esse anfípodo ancestral tenha surgido em águas de clima temperado e então se deslocado em direção à região norte da América do Sul, somente após a separação das Américas dos demais continentes

(GONZÁLEZ, 2001). A colonização da água doce teria ocorrido na época dos grandes mares epicontinentais, com a conquista dos ambientes dulcícolas Sul-Americanos e posteriormente se dispersando para a América Central e do Norte. Análises moleculares sugerem que as espécies que ocorrem nas Américas do Norte e Central tenham se diversificado antes do surgimento do Istmo do Panamá, no médio Mioceno, a cerca de 11 milhões de anos atrás (WITT; HEBERT, 2000).

A ocupação de *Hyalella* pela América do Sul teria ocorrido sem que essa estivesse totalmente formada geologicamente. O fato de populações do gênero serem encontradas a oeste da Cordilheira dos Andes no Chile, sugere que esses crustáceos colonizaram o continente antes do soerguimento da mesma. Além disso, os períodos de glaciações subsequentes teriam sido um dos principais eventos vicariantes de diversas populações, com o consequente surgimento de novas espécies de *Hyalella* (GONZÁLEZ, 2001; GONZÁLEZ; WATLING, 2003b). Contudo, nunca nenhum estudo específico acerca da biogeografia e ocupação do gênero foi realizado, de forma que análises moleculares abordando a filogenia do grupo seriam ideais para esclarecer tais questões.

Como já foi abordado anteriormente, estudar a dinâmica populacional e a ecologia desses anfípodos de água doce possibilita sua utilização em estudos de bioindicação, ecotoxicologia e na criação de protocolos de avaliação de qualidade ambiental de ecossistemas dulcícolas. Além disso, diversas características desses organismos possibilitam que sejam ideais no estudo da biogeografia e em programas de conservação e preservação.

Por não possuírem um estágio de vida dispersor, já que apresentam desenvolvimento direto, sem um estágio larval, as espécies de anfípodos de uma forma geral não possuem uma ampla distribuição geográfica (BARNARD; KARAMAN, 1983). Para as espécies de água doce, o endemismo parece ser ainda mais comum, principalmente com relação às que ocorrem em lagos, lagoas e ambientes subterrâneos (VÄINÖLÄ et al., 2008). Assim, por não se dispersarem por longas distâncias e por possuírem uma grande riqueza de espécies, os hyalellídeos são ideais para o estudo da biogeografia, pois sua atual distribuição pode refletir e inferir sobre os eventos biogeográficos históricos que moldaram e culminaram nas atuais fisionomias terrestres, bem como na idade desses processos.

Diversos trabalhos sobre a biogeografia de *Hyalella* na América do Norte já foram realizados, e a grande maioria se baseia em análises moleculares, como os de filogenia, filogeografia e os de identificação e separação de espécies crípticas e simpátricas, utilizando principalmente os genes 16S, COI e 28S (WITT; HEBERT, 2000; WITT; BLINN; HEBERT, 2003; WELLBORN; COTHRAN, 2004; WELLBORN; COTHRAN; BARTHOLF, 2005; WITT; THRELOFF; HEBERT, 2006; WITT; THRELOFF; HEBERT, 2008). Estudos genéticos têm se mostrado ferramentas essenciais na taxonomia e descrição da diversidade de táxons problemáticos, bem como na criação de programas de conservação de ambientes aquáticos de água doce ameaçados (WITT; THRELOFF; HEBERT, 2006)

Infelizmente ainda não existem estudos sobre a biogeografia e a filogenia molecular das espécies de *Hyalella* na América do Sul. Dessa forma, informações sobre a distribuição do gênero e seu uso na conservação e preservação de ecossistemas dulcícolas nesse continente ainda é ausente e pouco explorada.

2.5.1 *Hyalella* no Brasil

Das 68 espécies válidas de *Hyalella*, 23 ocorrem no Brasil. No país, atualmente, o gênero tem registro somente para as regiões sudeste e sul. Na região sudeste ocorrem 12 espécies, e dessas, seis ocorrem em Minas Gerais, seis no estado de São Paulo e duas no estado do Rio de Janeiro. A região sul possui o registro de 11 espécies, duas no Paraná e nove no Rio Grande do Sul, estado com a maior riqueza conhecida no país (BUENO; RODRIGUES; ARAUJO, 2014; CARDOSO et al., 2014; RODRIGUES; BUENO; FERREIRA, 2014).

As espécies brasileiras de *Hyalella* ocorrem em quase todos os biomas do país, como a Caatinga, Mata Atlântica, Cerrado e Pampa. Suas populações podem ser encontradas tanto em locais preservados quanto em regiões antropizadas, o que infelizmente é a realidade da maioria. Apenas algumas das espécies conhecidas atualmente ocorrem em áreas de conservação, como APAs, Parques Nacionais, interior de cavernas e áreas de difícil acesso, o que contribui com a preservação dessas populações (BUENO; RODRIGUES; ARAUJO, 2014).

Os hyalellídeos brasileiros podem ser encontrados em nascentes, rios de primeira e segunda ordem, áreas úmidas, lagoas e sistemas subterrâneos. Em geral, esses corpos d'água possuem baixa velocidade ou são lênticos, o que permite que os animais nadem na coluna d'água sem serem levados pela correnteza. Além disso, as espécies epígeas sempre estão associadas a plantas aquáticas, algas ou ao sedimento, utilizando-os como abrigo e fonte de alimento. Dessa forma, a presença da vegetação marginal nos ambientes aquáticos se torna vital para a preservação e manutenção das populações desses anfípodos (BUENO; RODRIGUES; ARAUJO, 2014).

No Brasil, a maior parte das espécies ocorre em ambientes epígeos, mas algumas poucas estão confinadas a ambientes subterrâneos. Até o momento, todas as seis espécies hipógeas de *Hyalella* conhecidas para o Brasil são troglóbias. Elas apresentam grandes adaptações morfológicas a esses habitats, como ausência de olhos, alongamento de apêndices e aumento no tamanho e número de estruturas sensoriais (CARDOSO et al., 2014; RODRIGUES; BUENO; FERREIRA, 2014).

Hyalella, apesar de ser o único gênero epígeo de anfípodos dulcícolas no país, ainda é pobremente estudado quanto à sua biologia, ecologia, distribuição, e sua diversidade certamente está subestimada, pois diversas novas espécies foram descritas nos últimos anos. Além disso, diversas regiões do Brasil ainda carecem de coletas, como as regiões Centro-Oeste, Nordeste e Norte (BUENO; RODRIGUES; ARAUJO, 2014).

Em um trabalho ainda não publicado, Rodrigues (2011) analisou a distribuição geográfica de *Hyalella* em áreas úmidas do Rio Grande do Sul. Os resultados mostraram (além de uma enorme diversidade de espécies, algumas delas sendo novas) que as populações, de forma geral, ocorriam com maior frequência nas áreas que possuíam água ao longo do ano, bem como nas que apresentavam algum tipo de vegetação aquática natural. Em áreas antropizadas, como plantações de arroz e aquelas onde a vegetação marginal foi suprimida, não foram encontradas populações dos anfípodos, evidenciando a importância de um ambiente equilibrado e conservado para a sobrevivência desses animais.

Devido às grandes reservas de água doce e à diversidade de *Hyalella*, o Brasil possui um enorme potencial para a realização de estudos ecológicos com anfípodos dulcícolas. Apesar disso, apenas alguns poucos trabalhos foram conduzidos até hoje. Com relação ao ciclo de vida, dinâmica populacional, ecotoxicologia e aspectos ecológicos, pouco é conhecido sobre as espécies brasileiras, de forma que artigos publicados foram somente sobre duas espécies simpátricas do Rio Grande do Sul: *H. castroi* González, Bond-Buckup & Araujo, 2006 e *H. pleoacuta* González, Bond-Buckup & Araujo, 2006 (CASTIGLIONI & BOND-BUCKUP, 2007; CASTIGLIONI et al., 2007; CASTIGLIONI & BOND-BUCKUP, 2008; DUTRA et al., 2007, 2011).

Apesar disso, esse cenário vem mudando nos últimos anos, de forma que alguns trabalhos acadêmicos, como monografias e dissertações, tem sido realizados acerca da ecologia das espécies do sudeste. Torres (2012) e Pereira (2014) estudaram a dinâmica populacional e o ciclo de vida de *H. carstica* Bastos-Pereira & Bueno, 2012 e *H. longistila* (Faxon, 1876), respectivamente, em Minas Gerais. Essas autoras puderam verificar que as espécies do sudeste estão submetidas a diferentes pressões ambientais quando comparadas às espécies do sul, como a estiagem durante o inverno, picos de reprodução após a época de seca, menor fecundidade das fêmeas, maior investimento no tamanho dos ovos, entre diversos outros fatores.

Essas diferenças observadas salientam a importância de se conhecer a ecologia desses anfípodos em ambientes e regiões distintos, demonstrando que esses animais estão extremamente adaptados ao ambiente em que vivem, permitindo seu uso em programas regionais de biomonitoramento e conservação.

Diversos morfotipos de *Hyalella* que ocorrem no Brasil e ainda não foram formalmente descritos são extremamente parecidos morfologicamente, sendo difícil separá-los usando apenas a taxonomia (observação pessoal). Para que não ocorram erros de identificação e de futuras descrições de espécies, é necessário a realização da filogenia molecular de todas as espécies brasileiras conhecidas até então, pois assim não existirão dúvidas sobre a validade desses táxons.

Como exemplo, pode-se citar a controversa espécie *H. curvispina* Shoemaker, 1942, que segundo Grosso e Peralta (1999) possui uma ampla distribuição por toda a América do Sul, incluindo o Rio Grande do Sul, Uruguai, parte da Argentina e do Chile. Contudo, González e Watling (2003b) afirmam que essa distribuição proposta por Grosso e Peralta (1999) é errada, tratando-se de um grande complexo de diferentes espécies. Além disso, González e Watling (2003b) descrevem diversas novas espécies a partir do material de Grosso e

Peralta (1999), que supostamente tratava-se apenas de *H. curvispina*. Contudo, os próprios autores afirmam que essas descrições realizadas por eles devem ser confirmadas com dados moleculares, a fim de se resolver o problema dos complexos de espécies.

2.5.2 Os complexos de espécies de *Hyalella*

Um complexo de espécies pode ser definido como um grupo constituído por espécies morfologicamente semelhantes entre si, sendo praticamente impossível diferenciá-las usando somente a taxonomia, no entanto, são geneticamente diferentes (STOCK; PLATVOET, 1991). Geralmente, as espécies que compõe um complexo, como é o caso de *Hyalella*, ocupam uma mesma região geográfica e/ou ocupam uma mesma bacia hidrográfica.

Dentro de *Hyalella*, as espécies que compõem os complexos são extremamente semelhantes na morfologia, de forma que muitas vezes são confundidas umas com as outras. Isso impossibilita mapear a real distribuição geográfica das espécies, realizar estudos ecológicos comparando diferentes populações, bem como criar medidas de conservação. Muitas vezes, espécimes são identificados de forma errônea e outras diversas já foram descritas e posteriormente consideradas como sinônimos juniores (GONZÁLEZ, 2001; WITT; THRELOFF; HEBERT, 2008).

Através de análises moleculares já foi confirmada a existência do complexo "*H. azteca*", que abrange 13 espécies das Américas do Norte e Central (WELLBORN; COTHRAN, 2004; WELLBORN; COTHRAN; BARTHOLF, 2005; WITT; THRELOFF; HEBERT, 2008). González (2001), baseando-se apenas na morfologia, também sugere a existência dos complexos "*H. patagonica*" e "*H. curvispina*", porém, nunca foram realizadas análises moleculares que confirmem sua hipótese. O complexo "*H. patagonica*" englobaria as espécies que ocorrem no extremo sul da América do Sul e outras que ocorrem ao longo da Cordilheira dos Andes. Já o complexo "*H. curvispina*" incluiria espécies presentes no Uruguai, Argentina e sul do Brasil. No entanto, espécies descritas recentemente e que possuem características morfológicas e ocorrência nessas regiões se enquadrariam no complexo, de forma que o mesmo precisa ser revisto.

A melhor opção para solucionar os complexos tem sido a utilização de análises moleculares, pois elas conseguem diferenciar geneticamente as espécies que são morfologicamente semelhantes. No momento, estudos sobre a filogenia molecular, filogeografia e biogeografia estão restritos somente às espécies da América do Norte. Como exemplo, pode-se citar o trabalho de Witt e Hebert (2000). Ao estudarem populações de *Hyalella* dos EUA consideradas morfologicamente como sendo *H. azteca*, os autores descobriram através de dados moleculares que se tratavam de pelo menos 10 novas espécies, todas escondidas sob a forma de espécies crípticas.

Além disso, devido à alta endemicidade, as espécies de *Hyalella* podem ajudar a criar programas de conservação de ambientes aquáticos de água doce ameaçados. Witt, Threloff e Hebert (2006) demonstraram esse potencial para conservação ao verificarem a existência de 35 novas espécies de *Hyalella* em uma área relativamente pequena dos desertos dos estados da Califórnia e Nevada, nos EUA. Praticamente todas as novas espécies são endêmicas das nascentes nos desertos, habitats extremamente ameaçados na região devido a sobre-exploração de água subterrânea. Assim, praticamente cada ponto coletado no trabalho citado apresentava uma nova espécie de *Hyalella*, o que contribuiu para a criação de medidas de conservação de ambientes aquáticos na região (WITT; THRELOFF; HEBERT, 2006).

Nenhuma das novas espécies encontradas nos últimos trabalhos citados foi descrita até o momento, principalmente pela falta de taxonomistas especialistas no grupo como pela grande dificuldade de identificação das espécies. Estima-se que existam pelo menos 500 espécies novas de *Hyalella* a serem descobertas somente na América do Norte, o que tornaria o gênero o mais abundante entre os anfípodos de água doce na região (VÄINÖLÄ et al., 2008).

Nenhum tipo de estudo filogenético, biogeográfico ou que estime a diversidade foi realizado até o momento com as espécies de *Hyalella* da América do Sul, apesar do continente possuir grande parte da diversidade do gênero. A existência dos complexos na América do Sul bem como de espécies crípticas ainda não foi comprovada, de forma que a real diversidade de *Hyalella* nesse continente ainda é um mistério. Trabalhos que utilizem técnicas moleculares a fim de se estudar a filogenia, a biogeografia e a distribuição das espécies de *Hyalella* no Brasil certamente serão uma grande ferramenta para melhor compreender sua diversidade, bem como ajudar a preservar os únicos anfípodos dulcícolas epígeos no país.

3 OBJETIVOS

O objetivo dessa tese foi conhecer a filogenia molecular do gênero de anfípodos de água doce *Hyalella*, sua biogeografia na América do Sul bem como aspectos da estrutura populacional de distintas espécies em diferentes latitudes. Com esses resultados será possível auxiliar na criação de estratégias de conservação desses animais bem como dos ecossistemas dulcícolas onde vivem.

3.1 Objetivos específicos

a) Atualizar o número de espécies que ocorrem no país, bem como sua distribuição;

b) Testar se as espécies de *Hyalella* que ocorrem no Brasil formam um grupo monofilético;
c) Verificar se a análise morfológica é suficiente para delimitar as espécies de *Hyalella*;
d) Confirmar se o complexo "*H. curvispina*" existe na América do Sul;
e) Estimar o tempo de diversificação das espécies brasileiras de *Hyalella* e a biogeografia do gênero no país;
f) Conhecer e comparar a estrutura populacional de quatro espécies de *Hyalella* no Brasil em diferentes latitudes, bem como o período reprodutivo e abundância das populações;
g) Analisar e comparar os aspectos populacionais dessas espécies, como razão sexual, distribuição em frequências de classes de tamanho, tamanho médio dos animais, maturidade sexual e fecundidade;
h) Verificar se os parâmetros ambientais que variam ao longo das latitudes influenciam na estrutura das populações de *Hyalella* e seus aspectos;
i) Futuramente, com os resultados obtidos nessa tese, auxiliar na criação de programas de conservação de ecossistemas de água doce no Brasil.

4 HIPÓTESES

a) A diversidade de espécies de anfípodos dulcícolas no Brasil e na América do Sul é subestimada e está escondida na forma de espécies crípticas;
b) As espécies brasileiras de *Hyalella* não formam um grupo monofilético;
c) A identificação morfológica das espécies de *Hyalella* não reflete a real história evolutiva do gênero;
d) As espécies que ocorrem geograficamente próximas ou que ocorrem na mesma bacia hidrográfica estarão unidas na árvore filogenética;

e) O complexo de espécies "*H. curvispina*" existe na América do Sul, abrangendo espécies do Brasil e de outros países;
f) O gênero *Hyalella* se diversificou após a separação das Américas da Laurásia e Gondwana e antes do surgimento do Istmo do Panamá;
g) A origem de *Hyalella* seria a América do Sul, de forma que a dispersão do gênero para a América do Norte tenha sido antes do surgimento do Istmo do Panamá;
h) Espécies de anfípodos de água doce que ocorrem em baixas latitudes do Brasil apresentam padrões de história de vida e estrutura da população similares entre si, e espécies de altas latitudes no país apresentam padrões similares aos anfípodos do Hemisfério Norte;
i) Os parâmetros ambientais influenciam nos aspectos populacionais das espécies de *Hyalella*. Ambientes mais quentes e próximos dos trópicos aumentam a densidade das populações, a fecundidade, estendem o período reprodutivo e reduzem o tamanho corporal. Ambientes mais frios e em latitudes mais altas diminuem a densidade populacional, a fecundidade e o período reprodutivo e aumentam o tamanho corporal dos indivíduos.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1

Molecular phylogeny of the Brazilian species of the genus *Hyalella* (Crustacea, Amphipoda): implications for freshwater environments conservation

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ABSTRACT

Nowadays, conservation biology confronts two major challenges: the number of described species be much lower than the actual biodiversity and the existence of cryptic species. Infer the molecular phylogeny has a great potential for conservation purposes by clarifying species boundaries and phylogenetic relationships, aiding to reconstruct evolutionary and biogeographic histories. In this study we examine *Hyalella*, a freshwater crustacean endemic for Americas, with several cryptic species. Here, we inferred the first molecular phylogeny of the genus in South America and check the existence of a species complex. We also infer the diversification times and historical biogeography of some *Hyalella* species. One nuclear (H3) and two mitochondrial genes (16S and 12S) have been chosen to elucidate relationships, reconstructed through Maximum Likelihood and Bayesian analyzes. Our results revealed high levels of cryptic diversity and endemism, a strikingly number of 20 new species and the existence of the species complex "*H. curvispina*". The nucleotide divergences among species and provisional species ranged from 0.1% to 30.8%, and the clustering in the phylogram is not related to the river basin and geographic distribution. The species of the subgenus *Austrohyalella* did not form a monophyletic group. Active dispersion and vicariance must have influenced the actual distribution of *Hyalella,* which dispersed from South America to North America. Our Bayesian divergence dating analysis revealed a Eocene origin for *Hyalella*, with a peak of diversification in Miocene. These results have important implications for the conservation of life in freshwater ecosystems in South America, as these crustaceans are endemic, bioindicators of water quality and their habitat are extremely threatened.

Keywords: Amphipods, divergence time, Hyalellidae, mitochondrial DNA, Peracarida

1 INTRODUCTION

Freshwater environments are essential for the survival of countless organisms, although, they are considered the most threatened ecosystems in the planet and usually they are not included in conservation programs (ABELL, 2002; HIGGINS et al., 2005; ABELL et al., 2008). Aquatic environments in South America are transboundary ecological units and its basins are large and drain massive volumes of water. Nevertheless, the region is very lacking in information on the ecology, biology and taxonomy of freshwater organisms (ABELL et al., 2008; BARLETTA et al., 2010).

Brazil comprises about 15% of all species in the world and the aquatic ones are certainly the most unknown. Only 11% of the conservation research in the country address freshwater environments and usually solely vertebrates are studied (AGOSTINHO; THOMAZ; GOMES, 2005). The increase of the biodiversity loss in Brazilian inland waters require the urgent creation of protected areas as well as ecological and taxonomic studies about the freshwater taxa (AGOSTINHO; THOMAZ; GOMES, 2005).

Understanding the species and populations boundaries is essential for generating conservation strategies. However, conservation biology confronts two major challenges: the number of described species be much lower than the actual biodiversity and the fact that many of these are morphologically cryptic, i.e., they can only be delimited using molecular genetics (HEBERT et al., 2003; WITT; THRELOFF; HEBERT, 2006). This tool has a great potential for conservation purposes by clarifying species boundaries and the phylogenetic relationships, aiding to reconstruct evolutionary and biogeographic histories and estimating their real geographical distribution (KNOWLTON, 2000; HEBERT et al., 2003).

Cryptic species and species complex have been extensively reported for crustaceans. In some freshwater taxa the diversity is still very underestimated and is hidden in the form of cryptic species, making difficult the work of taxonomists, ecologists and conservationists (JARA; PÉREZ-LOSADA; CRANDALL, 2003; WELLBORN; COTHRAN, 2004; BUHAY; CRANDALL, 2005; WITT; THRELOFF; HEBERT, 2006; SEIDEL; LANG; BERG, 2009; PILEGGI; MANTELATTO, 2010; de CARVALHO; PILEGGI; MANTELATTO, 2013).

A good model of this situation is the freshwater amphipod crustacean *Hyalella* Smith, 1874, which occurs exclusively in freshwater only in Americas, from Southern Patagonia and Falkland Islands to central Canada (BUENO; RODRIGUES; ARAUJO, 2014). Currently, there are 68 species described for the genus and 52 of them occur in South America. Brazil has the greatest diversity of the world with 23 species, but this number certainly will increase (RODRIGUES; BUENO; FERREIRA, 2014; SOUCEK et al., 2015). Species of *Hyalella* are among the organisms most commonly used in environmental quality tests as some of them are considered bioindicators, presenting high sensitivity for contaminants and environmental impacts (PILGRIM; BURT, 1993; DUAN; GUTTMAN; ORIS, 1997; MORRIS; COLLYARD; MEYER, 2002; WILCOXEN; MEIER; LANDRUM, 2003; GUST, 2006; DING et al., 2011).

Hyalella presents a complicated taxonomic history and currently includes three subgenera, which are identified according to morphology and geographical distribution: *Austrohyalella* is the most primitive subgenus and is restricted to Argentina, Chile, Patagonia and Falkland Islands; *Mesohyalella* includes species with a phyletically intermediate condition and are distributed along South America, with the highest diversity in Brazil; *Hyalella* is the nominate subgenus and is restricted to Central and North America

(BOUSFIELD, 1996). Bousfield (1996), based entirely on morphological observations, suggests that the subgenus *Hyalella* have proliferated only after the raise of the Isthmus of Panama, about 3 million years ago, following the emigration of a presumed mesohyalellid ancestral. However, Witt and Hebert (2000) when performed molecular analyzes found that this subgenus may be diversified before the formation of the Isthmus, as early as 11 million years ago, in the mid-Miocene.

Several species of *Hyalella* have been described for the entire American continent since the research of Bousfield (1996) (CARDOSO et al., 2014; RODRIGUES; BUENO; FERREIRA, 2014; COLLA; CÉSAR, 2015; SOUCEK et al., 2015) and the confirmation if the subgenera are monophyletic has never been tested. The establishment of these subgenera based only on morphology and geographic distribution is fragile, as there are species complex within the genus and the convergent evolution and parallelism of morphological characters seems to be common in freshwater amphipods (BARNARD, 1974; CULVER; JERNIGAN; O'CONNEL, 1994; SHERBAKOV et al., 1998; TRONTELJ; BLEJEC; FIŠER, 2012).

Many studies have demonstrated the existence of the large North American species complex "*H. azteca*" (HOGG et al., 1998; MCPEEK; WELLBORN, 1998; DUAN et al., 2000; WITT; HEBERT, 2000; WITT; BLINN; HEBERT, 2003; WELLBORN; COTHRAN; BARTHOLF, 2005; WITT; THRELOFF; HEBERT, 2006). Witt, Threloff and Hebert (2006) suggest that due to the high endemism (as most species are known only for single water body) *Hyalella* may be the most diverse amphipod of North America, possibly with 500 or more taxa, presenting a great potential to be used in conservation programs. Morphological analysis indicate also the existence of the species complex "*H. curvispina*" in South America, which occurs from Falkland Islands, Patagonia, Argentina, Chile, Peru, Uruguay to the Southeast Brazil (BOUSFIELD, 1996; STOCK; PLATVOET, 1991).

The great species diversity of *Hyalella*, the existence of a species complex and its supposed origin in South America reinforce the need to perform molecular studies using the species from the region. The molecular phylogeny of *Hyalella,* the relationships among its species and when their dispersion occurred across the continent can aid to understand the evolutionary and biogeographic history of the genus.

In this study we verify if the Brazilian species of *Hyalella* (*Mesohyalella*) form a monophyletic group, by inferring the first molecular phylogeny of the genus in South America, using mitochondrial and nuclear DNA sequences. Furthermore, we also investigate the existence of the "*H. curvispina*" species complex and we estimate a timescale for the diversification in the genus and its biogeographic history.

2 MATERIAL AND METHODS

2.1 Sample collection and morphological identification

We performed samples in two countries of South America: Brazil and Uruguay and also included in our analyses specimens deposited in scientific collections from Argentina. The material from Uruguay and Argentina was used here to study the existence of the species complex "*H. curvispina*", as described below.

The species of *Hyalella* (*Mesohyalella*) that occur in Brazil were collected from several localities in South and Southeast regions, the only two where the genus is currently found in the country. Among the 25 species described for Brazil, six occur in subterranean environments (CARDOSO et al.,

2014; RODRIGUES; BUENO; FERREIRA, 2014) and were not included in this study. Unfortunately, not all 19 Brazilian epigean species were found in their type-locality, mainly due to the disappearance of the water body or because the population is probably locally extinct.

We collected *H. curvispina* in its type-locality in Uruguay and we included specimens of this species from Uruguay and Argentina, which were deposited in the Crustaceans Collection of Universidade Federal de Lavras (CCUFLA), in order to confirm the existence of the complex "*H. curvispina*". We also included *H. araucana*, *H. kochi* (*Austrohyalella*) from Argentina and *H. azteca* (*Hyalella*) from USA, all from scientific collections, to verify if the subgenera of *Hyalella* are monophyletic.

The amphipods were sampled using a small hand net and all specimens were stored in absolute ethanol from their time of capture until the molecular analysis. Thus, we collected 13 known species from Brazil and more 21 were considered provisional species through morphological analyzes (one from Uruguay). All information about the material used in this research are listed in Table 1. All the populations used and/or sampled for this work are shown on Figures 1-3.

For each locality at least three male specimens were dissected under a stereoscope microscope. The morphological identification of the species was made through the montage of glycerol gel slides using the appendages of the specimens. The following morphological characteristics of *Hyalella* were used to perform the identification, according to Bueno et al. (2013) and Rodrigues, Bueno and Ferreira (2014): presence of dorsal flanges on pereons and pleonites; number of sternal gills tubular; number and arrangement of comb-scales and setae of uropods, telson and gnathopods; presence of a curved seta on inner ramus of uropod 1; and shape and size of propodus of gnathopod 1 and 2.

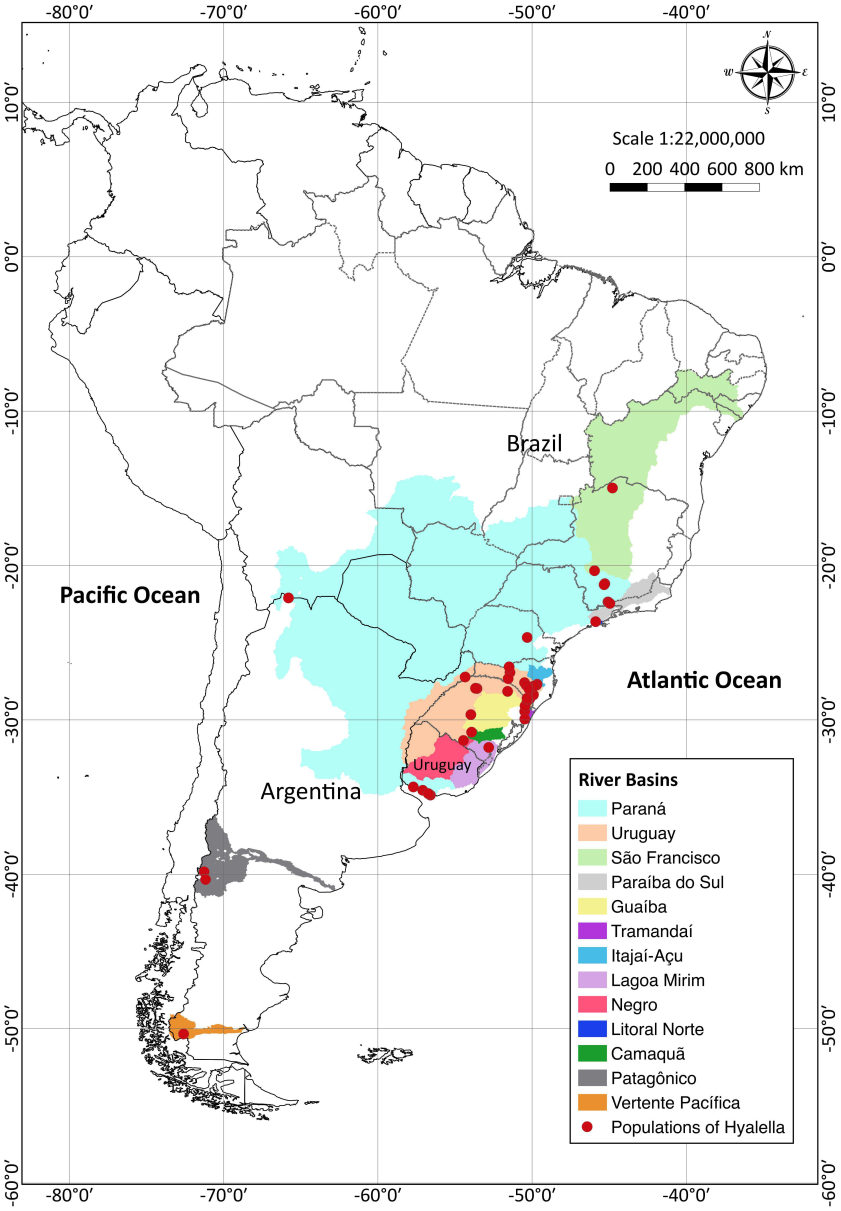

Figure 1 Map of South America showing the River Basins and the location of the populations of *Hyalella* studied in this work.

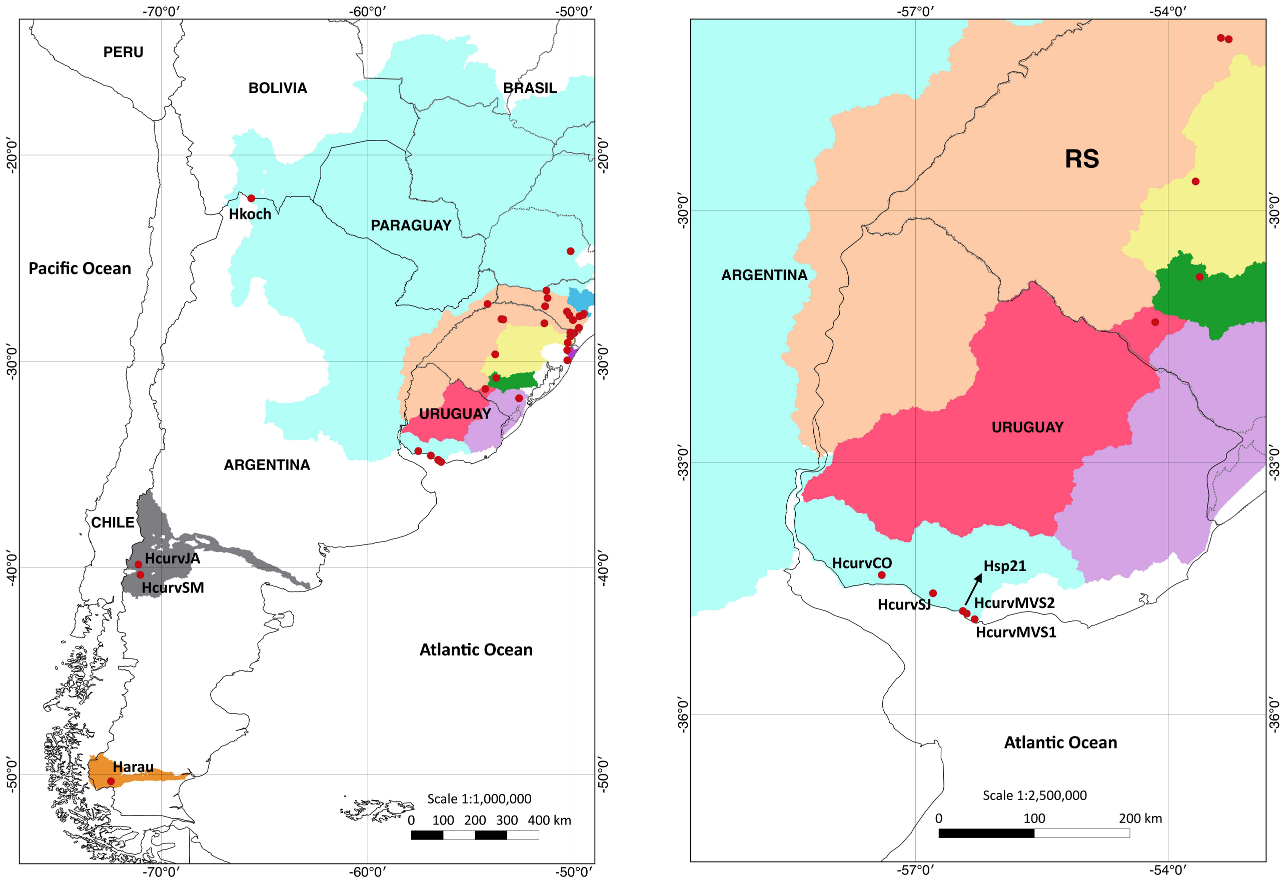

Figure 2 Maps showing the localities of the Argentinean (A) and Uruguayan (B) species of *Hyalella* studied in this work. The codes for each species are shown on Table 1.

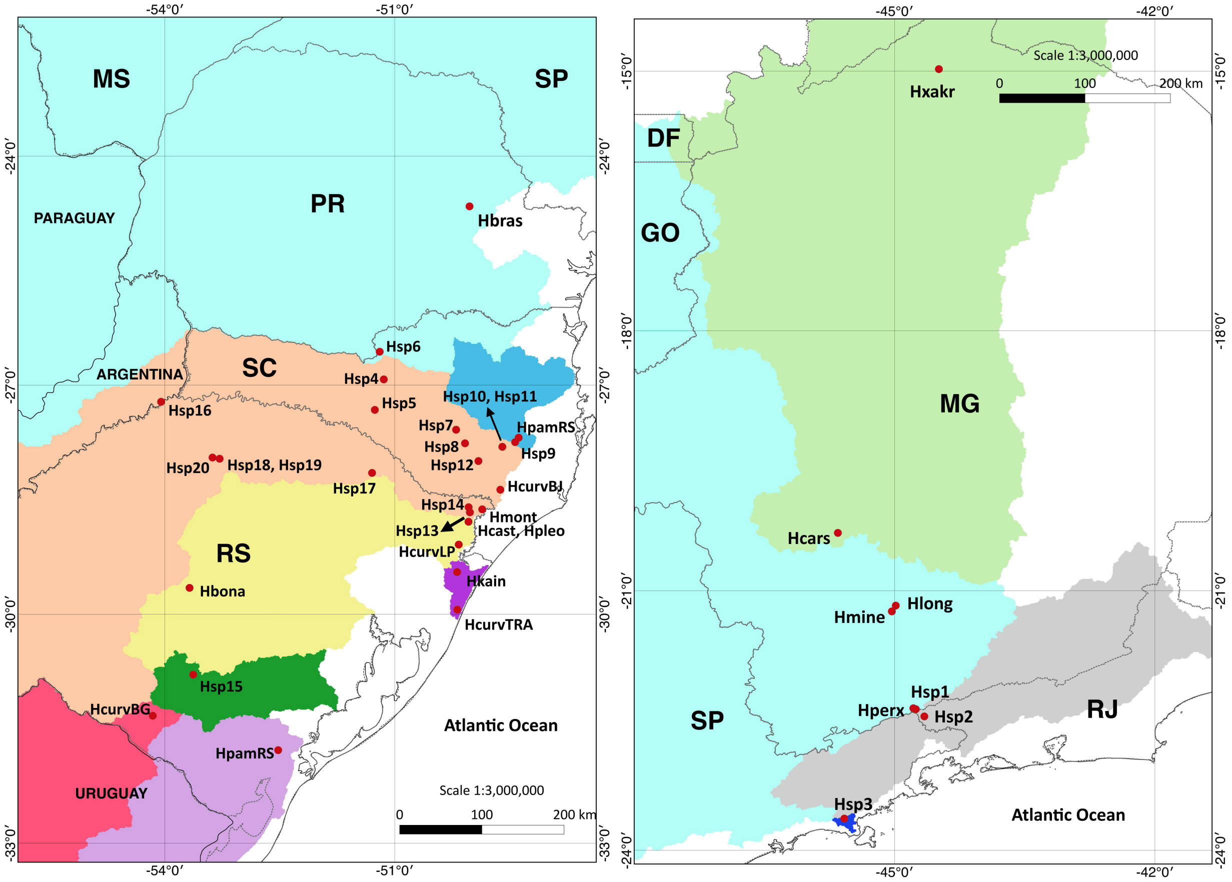

Figure 3 Maps showing the localities of the species of *Hyalella* from Southern (A) and Southeastern (B) Brazil studied in this work. The codes for each species are shown on Table 1.

Table 1 Nominal species of *Hyalella* with sample sizes (*N*), its collection sites, geographic coordinates and its localization in the map of the Figure 1. The acronyms refer to the Brazilian states: MG (Minas Gerais), RJ (Rio de Janeiro), PR (Paraná), SC (Santa Catarina) and RS (Rio Grande do Sul). The material obtained from scientific collections are indicated with an asterisk*. IPD: Intrapopulations pairwise nucleotide sequence divergences in percentage.

Nominal species (*N*)	**Collection site**	**Latitude/Longitude**	**Map locality**	**IPD (%)**
Hyalella xakriaba (10)	Peruaçu National Park/MG	14°58′36.5″S/44°26′36″W	Hxakr	0.00
Hyalella longistila (13)	Ijaci/MG	21°10′24″S/44°56′24.2″W	Hlong	0.00
Hyalella carstica (10)	Arcos/MG	20°19′59.6″S/45°36′25.3″W	Hcars	0.10
Hyalella minensis (13)	Lavras/MG	21°13′84″S/44°58′66″W	Hmine	0.00
Hyalella pernix (10)	Itatiaia National Park/RJ	22°21′32.1″S/44°44′13.9″W	Hperx	0.00
*Hyalella brasiliensis** (7)	Castro/PR	24°39′31.3″S/49°58′16.6″W	Hbras	0.00
Hyalella bonariensis (8)	Silveira Martins/RS	29°39′23.5″S/53°37′37.3″W	Hbona	0.00
Hyalella kaingang (3)	São Francisco de Paula/RS	29°27′S/50°08′W	Hkain	0.00
Hyalella montenegrinae (7)	São José dos Ausentes/RS	28°36.99′S/49°47.79′W	Hmont	0.10
Hyalella castroi (13)	São José dos Ausentes/RS	28°47.24′S/49°59.10′W	Hcast	0.10
Hyalella pleoacuta (13)	São José dos Ausentes/RS	28°47.24′S/49°59.10′W	Hpleo	0.10
Hyalella pampeana (5)	Pelotas/RS	31°46′57″S/52°28′10.9″W	HpamRS	0.30
Hyalella pampeana (7)	Alfredo Wagner/SC	27°41′21.4″S/49°19′48″W	HpamSC	0.60
Hyalella curvispina (16)	Lagoa do Passo/RS	29°5′23″S/50°06′45″W	HcurvLP	1.10
Hyalella curvispina (12)	Tramandaí/RS	29°56′30″S/50°07′50″W	HcurvTRA	0.20
*Hyalella curvispina** (11)	Bagé/RS	31°19′53″S/54°06′25″W	HcurvBG	0.00
*Hyalella curvispina** (5)	Bom Jardim da Serra/SC	28°22′14.4″S/49°34′9.9″W	HcurvBJ	0.10
*Hyalella curvispina** (9)	Junin de los Andes/Argentina	39°50′1.4″S/70°55′28.3″W	HcurvJA	0.10
*Hyalella curvispina** (9)	San Martin de Los Andes/Argentina	40°20′13.7″S/70°49′51.8″W	HcurvSM	2.40
Hyalella curvispina (6)	Montevideo (site 1)/Uruguay	34°51′59.9″S/56°15′09.3″W	HcurvMVS1	0.10
Hyalella curvispina (8)	Montevideo (site 2)/Uruguay	34°47′56.2″S/56°20′48.9″W	HcurvMVS2	0.10
Hyalella curvispina (8)	San José/Uruguay	34°33′25.7″S/56°44′51.2″W	HcurvSJ	0.00
Hyalella curvispina (10)	Colonia/Uruguay	34°20′26.3″S/57°21′22.9″W	HcurvCO	0.10

Table 1, continuation

Nominal species (*N*)	**Collection site**	**Latitude/Longitude**	**Map locality**	**IPD (%)**
*Hyalella araucana** (5)	Santa Cruz Province/Argentina	50°20′21″S/72°15′22″W	Harau	6.00
*Hyalella kochi** (7)	Jujuy Province/Argentina	22°05′53″S/65°27′16″W	Hkoch	8.60
*Hyalella azteca** (3)	Texas/USA	30°38′31.20″N/96°31′4.8″W	-	0.30
Hyalella sp. 1 (11)	Itatiaia National Park/MG	22°22′13.3″S/44°42′32.6″W	Hsp1	0.00
Hyalella sp. 2 (6)	Itatiaia National Park/MG	22°27′11.5″S/44°36′38.93″W	Hsp2	0.10
Hyalella sp. 3* (8)	Estação Biológica de Boracéia/SP	23°37′59″S/45°31′59″W	Hsp3	0.20
Hyalella sp. 4 (10)	Rio das Antas/SC	26°55′32.1″S/51°05′26.7″W	Hsp4	0.00
Hyalella sp. 5 (11)	Campos Novos/SC	27°19′29.1″S/51°12′28.1″W	Hsp5	0.00
Hyalella sp. 6 (4)	Matos Costa/SC	26°33′47.2″S/51°08′38.9″W	Hsp6	0.10
Hyalella sp. 7 (8)	Palmeira/SC	27°35′05.6″S/50°08′48.0″W	Hsp7	0.00
Hyalella sp. 8 (10)	Bocaina do Sul/SC	27°45′45.6″S/50°01′49.4″W	Hsp8	0.10
Hyalella sp. 9 (9)	Alfredo Wagner/SC	27°44′48.9″S/49°22′33.9″W	Hsp9	0.10
Hyalella sp. 10 (4)	Bom Retiro/SC	27°48′30.1″S/49°32′35.1″W	Hsp10	0.00
Hyalella sp. 11 (2)	Bom Retiro/SC	27°48′30.1″S/49°32′35.1″W	Hsp11	0.00
Hyalella sp. 12 (11)	Urupema/SC	27°59′42.9″S/49°51′19.6″W	Hsp12	0.00
Hyalella sp. 13 (15)	São José dos Ausentes/RS	28°40.28′S/49°57.94′W	Hsp13	0.40
Hyalella sp. 14 (10)	São José dos Ausentes/RS	28°35.89′S/49°58.77′W	Hsp14	0.10
Hyalella sp. 15 (10)	Caçapava do Sul/RS	30°47′36.3″S/53°34′46.9″W	Hsp15	0.00
Hyalella sp. 16 (13)	Derrubadas/RS	27°13′6.89″S/53°59′52.4″W	Hsp16	0.00
Hyalella sp. 17 (3)	Esmeralda/RS	28°9′1.55″S/51°14′38.44″W	Hsp17	0.20
Hyalella sp. 18 (2)	Palmeira das Missões/RS	27°57′52.79″S/53°14′2.59″W	Hsp18	0.10
Hyalella sp. 19 (3)	Palmeira das Missões/RS	27°57′54.3″S/53°14′03.09″W	Hsp19	0.10
Hyalella sp. 20 (3)	Palmeira das Missões/RS	27°56′56.80″S/53°19′37.3″W	Hsp20	0.10
Hyalella sp. 21 (10)	San José/Uruguay	34°46′06.3″S/56°23′33.8″W	Hsp21	0.00

2.2 DNA extraction, amplification and sequencing

All sequences obtained in this study were generated from our own extractions. Genetic vouchers were deposited in appropriate scientific zoological collections. Total genomic DNA was extracted from 2 to 16 individuals from each nominal species. DNA was extracted from the entire animal using the Qiagen DNeasy® Blood and Tissue Kit (Cat. No. 69582).

Targeted gene regions were amplified by means of the polymerase chain reaction (PCR): 16S, ribosomal subunit was amplified with the newly designed primers 16Samphi-f (5'-AGT GYA CAA ATT GCC CGT CAY TCT C-3') and 16Samphi-r (5'-ACA ADT TAT TAT GCT ACC TTA GCA C-3') (~650bps); 12S, ribosomal subunit was also amplified with the newly designed primers 12Samphi-f (5'-YTA CTT TGT TTC GAC TTR YC-3') and 12Samphi-r (5'-WTD GTG CCA GCW NYT GCG GTT-3') (~500bps); and for the H3 protein-coding gene we used the primers H3af (5'-ATG GCT CGT ACC AAG CAG ACV GC-3') and H3ar (5'-ATA TCC TTR GGC ATR ATR GTG AC-3') (~375bps, COLGAN et al., 1998).

PCR reactions were performed in 25 µl volumes containing 15 µl of ultrapure water, 2.5 µl of 10X PCR buffer, 1.5 µl of $MgCl_2$ (25mM), 1.25 µl of deoxyribonucleotide triphosphate mix (dNTPs, 10mM), 1.0 µl of each primer (10mM), 0.75 µl of Bioline Taq polymerase and 3 µl of extracted DNA. The thermal profile used for 16S and 12S was performed as follows: initial denaturation for 5 min at 96°C, followed by 10 cycles of 30 sec at 96°C, 60 sec at 55°C (decreasing annealing temperature by 1°C/cycle during these 10 cycles) and 60 sec at 72°C, followed again by 30 cycles of 30 sec at 96°C, 60 sec at 45°C and 60 sec at 72°C, with a final extension of 5 min at 72°C. Thermal cycling for H3: initial denaturation for 5 min at 96°C, followed by 30 cycles of 30 sec at 96°C, 45 sec at 50°C, and 60 sec at 72°C, with a final extension of 5 min at 72°C. PCR products were checked at electrophoresis with agarose gel (1%).

Successful PCRs were purified by using a 4 µl diluted mixture of ExoSap (USB). For the sequencing, 1 µl of the purified PCR was used in a 10 µl reaction [0.5 µl of BigDye terminator sequencing mixture (V3.1, ABI), 1.75 µl of a 5X buffer, 0.5 µl of a 10µM primer and 6.25 µl of ultrapure water]. The thermal profile of the cycling sequencing were the same for all genes: 30 cycles of 30 sec at 95°C, 30 sec at 50°C and 4 min at 60°C. An Applied Biosystems 2700 Thermal Cycler was used in PCR and cycle sequencing reactions. Following cycle sequencing, products were purified on a column of sephadex and sequences were run forward and reverse on an ABI3730 Genetic Analyzer (Applied Biosystems) in the Laboratories of Analytical Biology (LAB) - National Museum of Natural History, Smithsonian Institution. All sequences were confirmed by sequencing both strands and the consensus sequence for them was obtained using Geneious v7.1.5 (KEARSE et al., 2012).

2.3 Phylogenetic analyses and divergence time estimation

Sequences were cleaned and assembled using Geneious. To confirm the nonexistence of pseudogenes, we performed BLAST searches and the translating protein-coding sequences H3 were checked for indels and stop codons, comparing sequences among other freshwater amphipods. We built independent analysis for each gene and obtained the same overall topology.

Nucleotide sequences from each gene region were aligned in MAFFT v7.149b (KATOH; STANDLEY, 2013) under the global (G-INS-i) algorithm and default settings. After trimming for primer residue final alignments included 375, 494 and 644 base pairs for H3, 12S and 16S, respectively. In MESQUITE v. 3.04 (MADDISON; MADDISON, 2011), all genes were concatenated and partitioned for analysis. The final dataset included 417 individuals and 1,513 basepairs (3 genes). The model of evolution that best fit the data was determined for each gene region, with ModelTest v1.0.1 (POSADA; CRANDALL, 1998). The species *H. araucana* was used as outgroup. In accordance to Bousfield (1996), *H. araucana* was considered the most primitive species among the others of this work, either for belong to the subgenus *Austrohyalella* as to occur in the southernmost point of South America (Figure 1), estimated location of origin of the genus.

In order to estimate intra- and interspecific divergence rates, we calculated the genetic distances in MEGA v6.06 (TAMURA et al., 2013) using the uncorrected *p* distance. We assessed the extent of species diversity using a species screening threshold (SST), created by Hebert et al. (2004). This methodology was also used by Witt, Threloff and Hebert (2006) when studying the diversity of *Hyalella* in the southern Great Basin of California and Nevada, USA. The SST can be useful in recognizing provisional species in little studied groups, as *Hyalella* (WITT; THRELOFF; HEBERT, 2006). The SST set a 10 times the average intrapopulation nucleotides divergence, and despite being easily used, it overlooks recently diverged taxa (WITT; THRELOFF; HEBERT, 2006).

Maximum Likelihood (ML) analysis of the concatenated partitions were performed using RAxML Randomized Axelerated Maximum Likelihood v.8.2.4 (STAMATAKIS; HOOVER; ROUGEMONT, 2008). Likelihood settings followed the general time reversible model of evolution with proportion of invariable sites and gamma distribution (GTR + Γ+ I). ML was performed using 1,000 searches and 100 runs searching for the best-scoring ML tree. Confidence in the clade support was assessed using non-parametric bootstrap estimates with 1,000 pseudoreplicates and values >50% are shown on the resulting tree. Analysis were run on the high performance computing cluster Colonial One at

George Washington University. We also performed Bayesian analysis (BA) in MrBayes v3.2.4 (RONQUIST et al., 2012). Three independent BA analysis were run with four chains, one cold and three heated, starting , with a random tree, for 10,000,000 generations and sampling one tree every 1,000 generations. Using Tracer v1.6 (RAMBAUT et al., 2014) we graphically monitored all likelihood parameters, variances and scored means in order to ensure that the independent analyses converged on similar values. We discarded the first 20% of sampled generations and obtained a 50% majority-rule consensus tree. On the resulting tree, the posterior probabilities for individual clades obtained from separate analyses were compared for congruence and then combined and expressed as percentages.

Divergence times were estimated using BEAST v.1.8.2 (DRUMMOND et al., 2012). The input file was first generated in BEAUTi using the concatenated partitions of the three genes and the substitution model was the selected by ModelTest. We employed an uncorrelated lognormal relaxed clock model which were unlinked. Tree and substitution models were linked among partitions. For the tree prior we used the Yule speciation process with a random starting tree. Due to the poorly fossil record for Amphipoda (COLEMAN, 2006), with none belonging to *Hyalella*, we applied the Knowlton et al. (1993) mutation rate of mtDNA, derived of sequence divergences of malacostracan crustaceans. This mutation rate was also used by Witt and Hebert (2000) when studying the diversity and evolution of *Hyalella* in North America and it is estimated at 2.2 to 2.6% per 10^6 years. We performed two independent Markov chain Monte Carlo (MCMC) searches, each one with 100,000,000 generations, sampling one tree every 10,000 generations and a burn-in of 20%. Convergence between runs and analysis performance was checked with Tracer using effective sample size (ESS) scores. Then, we combined the resulting trees using TreeAnnotator v.1.8.2.

3 RESULTS

We obtained novel partial sequences of the 16S, 12S and H3 for all species included in this study. The concatenated data matrix contained 944 variable sites (62% of data set). Missing data were designated as a "?" in the alignment. The optimal models of evolution selected in ModelTest were K80 + $\Gamma + I$ (H3), TVM + $\Gamma + I$ (12S) and TrN + $\Gamma + I$ (16S).

The values of intrapopulation genetic divergence were very low. Most populations presented values lower than 0.1%, and many of those had no genetic divergence among the individuals sampled. However, populations of the species which occur in Argentina and USA were the ones that presented the highest genetic divergence values, reaching a maximum of 8.6% for *H. kochi* (Table 1).

The mean intrapopulation sequence divergence was 0.468% (*n*=47, SD=0.015%), resulting an SST of 4.68%. Pairwise nucleotide sequence divergences among the species and provisional species were as high as 30.8%, with an average of 19.46% ± 6.6%. The application of the SST indicated that (Table 2): the two sympatric species *H. pleoacuta* and *H. castroi*, as well as *Hyalella* sp. 13, constitute a single species (SST=0.1%); all populations sampled in Uruguay (country) are the same species - *H. curvispina* - as Montevideo site 1 it is the type locality of the species (SST values < 3.3%); "*H. curvispina*" from Lagoa do Passo/RS is the same species as *H. pampeana* from Pelotas/RS (SST=1.6%); *Hyalella* sp. 11 did not diverge of *H. pampeana* from Alfredo Wagner/SC (SST=1.6%); *Hyalella* sp. 9, *Hyalella* sp. 10 and *Hyalella* sp. 12 are apparently only one provisional species (SST values < 0.7%), as well as *Hyalella* sp. 4, *Hyalella* sp. 8 and *Hyalella* sp. 16 (SST values < 4.3%). Furthermore, the population of *H. curvispina* from Bagé/RS and *Hyalella* sp. 15 did not presented genetic divergence (SST values < 4.4%) from Uruguay populations and among themselves (SST=3.4%).

Table 2 Matrix of pairwise nucleotide sequence divergences (%) among species of *Hyalella* for the concatenated genes 16S, 12S and H3. Values lower than 4.68 are circled. MVS1: Montevideo, site 1, Uruguay; MVS2: Montevideo, site 2; SJ: San José, Uruguay; LP: Lagoa do Passo, Brazil; CO: Colonia, Uruguay; BG: Bagé, Brazil; BJ: Bom Jardim da Serra, Brazil; TR: Tramandaí, Brazil; SM: San Martin de Los Andes, Argentina; JA: Junin de los Andes, Argentina; PL: Pelotas, Brazil; AW: Alfredo Wagner, Brazil.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
1. *H. araucana*																						
2. *H. azteca*	29.8																					
3. *H. bonariensis*	28	28.4																				
4. *H. brasiliensis*	28.2	30.7	18.3																			
5. *H. carstica*	29.3	27.6	26	26.2																		
6. *H. castroi*	27.7	28.5	13.6	19.2	24.9																	
7. *H. curvispina* (MVS1)	24.4	24.5	4.4	18.4	23.8	13.5																
8. *H. curvispina* (MVS2)	26.5	26.2	4.5	17.3	24.2	13.3	0.1															
9. *H. curvispina* (SJ)	27.6	27.5	4.4	20	25.5	14.3	2.6	2.9														
10. *H. curvispina* (LP)	29.4	29	26.4	25.2	28	27	25.2	25.7	27													
11. *H. curvispina* (CO)	28	28.8	4.3	20	25.8	14	2.7	2.8	0.3	27												
12. *H. curvispina* (BG)	25.8	24.8	3.5	16.4	23.7	12.7	3.9	4.2	3.9	25	4											
13. *H. curvispina* (BJ)	26.8	25.3	13	17.3	24	7	11.5	11.8	13	26.3	13.5	10.8										
14. *H. curvispina* (TR)	26.4	27.8	14.7	18	25.3	15.2	12.9	12.8	13.9	27.3	13.9	13.4	13.3									
15. *H. curvispina* (SM)	27	26.9	18.7	23.2	25.9	20.2	18	17.9	19.3	25.6	19.4	17.8	19.4	19.8								
16. *H. curvispina* (JA)	30.6	26.3	15.5	16.2	21.2	15.7	15.4	15.3	15.3	22.5	15	15.2	16.4	14.9	12.3							
17. *H. kaingang*	22.6	23.4	12.8	18.8	24	13.2	10.8	11.7	12.5	26.7	13.3	10.2	11	12.7	18.9	15.3						
18. *H. kochi*	29.5	28.6	15	20.3	25.6	16.6	15.2	15	15.3	26	15.2	15	16.4	17.7	19	15.9	17					
19. *H. longistila*	30.8	28.7	25.7	28.2	21.9	26.5	24.5	24.4	26.7	28.2	26.9	22.8	26	27.9	28.3	24	25.5	26.3				
20. *H. minensis*	26.3	28.3	24.9	23.4	23.2	24.5	23.5	24	25.5	20.7	25.5	23.7	23.5	23.5	23.2	19.7	23.2	23.4	27.4			
21. *H. montenegrinae*	25.4	27	23	25.2	23.9	23.2	21.5	22.3	23.4	24.2	23.9	21.6	21.7	22.2	24.9	20	20	23.5	27.4	23.8		
22. *H. pampeana* PL	28.6	25.8	25.4	24.7	26.2	26.5	22.8	24.2	25.7	1.6	26.4	23	24	25.7	24.5	21.9	22.3	25.6	27.2	19.2	22.2	
23. *H. pampeana* AW	29.9	28.5	25.1	23	26	26.4	26	25.2	25.8	21	26	25.4	25	26	26	21.4	25.3	25.4	27	21.8	24.6	19.7
24. *H. pernix*	25.6	27.2	14.2	22	27.2	14.3	12.9	13	14	27.8	14.5	12.7	12.8	14	21	23	12.3	18.8	29.2	27	26.6	26.9
25. *H. pleoacuta*	27.2	28.7	13.7	19.7	25	0.1	13.7	13.4	14.5	27.2	14.3	12.8	7	15.2	20.6	15.7	13.6	16.7	26.8	24.8	23.5	26.7
26. *H. xakriaba*	28.8	28.2	26.6	28.2	9.4	28	25.9	25.8	27	28.7	27.2	24.4	26.4	26.9	27.7	23.2	26.7	27.4	23.2	26.6	27.6	27.6
27. *Hyalella* sp. 1	24.3	26.3	22.7	25.2	23.5	22.3	22	21.9	23.9	25.4	24	21	22	23.4	23	18	22.2	22.5	26.7	22.9	19.8	24
28. *Hyalella* sp. 10	25.7	25	14.4	19.3	23.8	14.7	12.2	13	14.3	25.8	14.5	12.2	13.2	13.9	18.7	16	11.3	17.8	27	23.5	21.7	23.7
29. *Hyalella* sp. 11	27.4	23.2	25.8	22.3	26.9	29	22.7	24.2	26.4	22	28	21	22.7	26.8	25.6	24	21	27.2	26.9	22.2	22.4	18.5
30. *Hyalella* sp. 12	25.5	26.6	14.4	18.5	23.6	14.2	13.2	13.6	14.4	25.7	14.5	13.2	13.3	13.7	18.4	15.7	12	17.5	26.9	23.7	22.6	24.5
31. *Hyalella* sp. 13	28.7	26.2	12.9	16.8	23.3	1.9	12.9	13.2	13	26.3	12.9	12	7.6	15	18.2	14.9	10.7	15.6	24.8	22.9	20.7	24.9
32. *Hyalella* sp. 14	24.8	28.7	21.8	24.6	23.7	21.2	21.7	21.4	22	24.5	22.3	20.4	21	22.2	25	17.8	21.9	21.7	26.2	24.6	9.4	23.8
33. *Hyalella* sp. 15	26.2	27	3.5	20	26.8	14.8	3.9	4.3	4.3	26.9	4.4	3.4	13.5	13.8	19.7	14.9	12.7	15.3	26.8	24.8	23	25.3
34. *Hyalella* sp. 16	27	27.4	6.4	19.5	26.8	16	6.6	6.8	6.2	26.9	6.2	7.2	14	14.9	20.5	18.3	13	17.5	26.8	25.7	24.7	25.7
35. *Hyalella* sp. 17	29	29.4	10.4	18.7	25.6	8.9	11.5	12	10.2	27.2	9.9	12	10.7	15	20	16.4	12.9	16.6	26.3	24	22.8	27
36. *Hyalella* sp. 18	25.3	23.3	15.4	18.8	23.7	14.6	12.3	14	14.4	25.9	14.9	13.6	12.6	13	18.7	16.8	9.9	17.9	26.3	23.9	20.7	22.7
37. *Hyalella* sp. 19	25.5	23	15.2	19.5	23.5	16.3	12.4	13.5	14.5	26	15.2	12.6	13.2	13	20	17.2	11.7	19.3	26.2	24.8	21.3	22.8
38. *Hyalella* sp. 2	25.4	26.4	13.8	19.9	24.6	11.7	13.6	13.8	14	27	14.3	13	10.3	13.3	19.2	13.9	12	16.6	27	23.9	22.6	25.2
39. *Hyalella* sp. 20	25.6	23.6	19.6	22.3	24.6	19.3	16.6	17.5	19.3	25.4	20.2	16.9	16.8	18.9	19.2	15.3	14.7	13.9	26.2	23	21.3	22
40. *Hyalella* sp. 21	26.5	28.3	5	20	25.9	14.5	0.7	0.6	3.3	26.7	3.3	4.4	13.6	13.6	19.6	15	13	15.4	26.9	24.8	23.6	25.7
41. *Hyalella* sp. 3	28.6	30	23	23.2	21.5	21.7	25.4	24.5	22.5	28.1	22.2	24.9	22.3	22	24.6	21.4	20.3	23.5	26.4	22.8	20.6	27.6

Table 2, continuation

	23	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**
1. *H. araucana*																		
2. *H. azteca*																		
3. *H. bonariensis*																		
4. *H. brasiliensis*																		
5. *H. carstica*																		
6. *H. castroi*																		
7. *H. curvispina* (MVS1)																		
8. *H. curvispina* (MVS2)																		
9. *H. curvispina* (SJ)																		
10. *H. curvispina* (LP)																		
11. *H. curvispina* (CO)																		
12. *H. curvispina* (BG)																		
13. *H. curvispina* (BJ)																		
14. *H. curvispina* (TR)																		
15. *H. curvispina* (SM)																		
16. *H. curvispina* (JA)																		
17. *H. kaingang*																		
18. *H. kochi*																		
19. *H. longistila*																		
20. *H. minensis*																		
21. *H. montenegrinae*																		
22. *H. pampeana* PL																		
23. *H. pampeana* AW																		
24. *H. pernix*	28.6																	
25. *H. pleoacuta*	26.7	14.3																
26. *H. xakriaba*	28	29	28.5															
27. *Hyalella* sp. 1	25.4	24.9	22.6	27.3														
28. *Hyalella* sp. 10	24.7	14.7	14.8	25.6	22.9													
29. *Hyalella* sp. 11	1.6	27.9	29.2	28	28.3	21.6												
30. *Hyalella* sp. 12	25.5	14.8	14.3	25.6	21.7	0.5	24.2											
31. *Hyalella* sp. 13	24.7	14	1.9	25	20.5	13.8	22	13.5										
32. *Hyalella* sp. 14	25	25.7	21.6	26.3	19.4	23.2	26.2	23.2	19.2									
33. *Hyalella* sp. 15	26.4	13.3	15	27.2	23.5	13.7	25.8	13.8	13.4	22.9								
34. *Hyalella* sp. 16	27.3	13.2	16	26.9	24.6	14.7	24.9	16	15.9	23.8	5.7							
35. *Hyalella* sp. 17	26	13	9.2	26.4	23.2	14.3	28	13.8	8.3	21	8.8	10.7						
36. *Hyalella* sp. 18	25	12.9	14.6	26.7	22.9	10.6	20.5	11.2	13.4	22.8	13.8	15.2	14.2					
37. *Hyalella* sp. 19	25.8	14.7	16.3	26.7	23.3	13.9	22.2	14.7	15.3	22.7	14.3	14.9	16.8	12.9				
38. *Hyalella* sp. 2	26.8	13.6	11.8	26	21.7	12.2	25.9	12.3	11.2	21.9	13.8	15	12.4	13	13.2			
39. *Hyalella* sp. 20	24.9	20.8	19.5	26.8	22	17.8	20.8	19	17	21.7	19	19.7	20.2	16.2	17.6	17.9		
40. *Hyalella* sp. 21	26.6	13.5	14.8	27.2	23.8	13.6	27	13.8	13.8	22.7	4.2	6.3	9.7	14	14.2	14	19.5	
41. *Hyalella* sp. 3	25	25.2	21.7	24.6	21.3	22.9	28.6	22.6	22.4	20.1	23.2	27.2	21.6	23.	22.4	21.7	23.9	22.9

Table 2, continuation

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
42. *Hyalella* sp. 4	27	28.2	6	19.9	26.7	15.5	6.3	6.2	6.4	26.7	6.2	5.8	14.6	14.7	20.6	16.3	14	15.9	27.5
43. *Hyalella* sp. 5	28.9	28.2	15.4	18.7	24.9	15.6	14	14	14.7	26.3	14.9	13.9	15.3	14	21	16.3	14	18.8	27
44. *Hyalella* sp. 6	24	25.8	14.6	20.2	24.8	14	13	13.3	15	27.7	15.3	13	13.5	13.5	19.7	14.9	11.5	17.5	28.2
45. *Hyalella* sp. 7	27.9	27	15.5	18.8	25	16.2	14	14	15	26.3	15.5	13.8	14.9	13.6	21.3	16.9	13.3	19	26.8
46. *Hyalella* sp. 8	27.3	27.9	6.2	19.5	26	15	6.2	6.2	6	26.6	5.9	6	14.2	14.8	20.2	16.8	13.7	15.9	27.3
47. *Hyalella* sp. 9	26	26.8	14.7	19.7	24.4	14.9	12.9	13.4	14.7	26	14.8	12.7	14	14.3	19.2	15.9	12.5	17.9	27.6

Table 2, continuation

	20	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**
42. *Hyalella* sp. 4	25	24.7	26	26.6	14.2	15.8	27	24.5	14.2	25.9	14	14	24.2	5.1	3.8	8.6	15.2	16.3
43. *Hyalella* sp. 5	25.7	23.8	25.8	27	16	15.6	27.7	24	15	29.5	14.7	14.8	23.9	15.2	16.4	16.7	14.6	8.5
44. *Hyalella* sp. 6	24.7	21.9	25.7	27	11.5	14	27.3	22.7	13	25.6	13.3	13.5	23.1	14.7	14.7	15.1	12.3	14
45. *Hyalella* sp. 7	26.3	23.3	25	27.6	15.5	16.3	27.3	24.9	14.6	27.4	14.6	15	23.9	15	16.5	17.2	14.4	8.2
46. *Hyalella* sp. 8	25	24.4	25.7	26	14	15.3	26.7	24	14.3	25.6	14	13.4	23.6	5.6	4.3	10.2	15.2	16
47. *Hyalella* sp. 9	24	23.2	25	25.5	15.2	15	26	23.3	0.1	24.6	0.6	14	23.6	14.3	15.4	14.3	11.6	14.9

Table 2, continuation

	38	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**
42. *Hyalella* sp. 4	15.5	20.5	6.7	23.8					
43. *Hyalella* sp. 5	14	20.7	14.6	23.4	16.2				
44. *Hyalella* sp. 6	12.7	17.8	14.1	22.3	15.8	15.5			
45. *Hyalella* sp. 7	13.6	19.7	15.4	23.7	17	5.1	15.4		
46. *Hyalella* sp. 8	15.5	20.3	6.6	23.2	0.3	15.9	15.6	16.8	
47. *Hyalella* sp. 9	12.6	19.3	13.8	23	14.6	15.1	13.7	15.1	14.7

The individual phylograms estimated for each gene inferred the same topology and are not shown in this study. The ML and BA analyzes for the concatenated dataset also resulted in the same topology (Figure 4), but with different degrees of support of some nodes. A total of 15 distinct groups can be visualized in the phylogram (Figure 4) and, in general, the clustering of species is not related to the river basin which they occur.

Surprisingly, species in the most basal clades (group 1 to group 5) are geographically very distant from the outgroup. Similarly, some species that occur geographically close did not result in monophyletic groups such as, e.g., *H. pernix*, *Hyalella* sp. 1 and *Hyalella* sp. 2, from Itatiaia National Park; the species from the region of São José dos Ausentes/RS (*H. castroi*, *H. pleoacuta*, *H. montenegrinae*, *Hyalella* sp. 13 and *Hyalella* sp. 14); and the species from Serra Geral/SC ("*H. curvispina*" de Bom Jardim da Serra, *Hyalella* sp. 7, *Hyalella* sp. 8 and *Hyalella* sp. 12). The resulting groups also do not reflect the morphology of the species, and these results will be discussed in a future paper.

The species of the subgenus *Austrohyalella* (*H. kochi* and *H. araucana*) did not form a monophyletic group (Figure 4) and the validity of this subgenus should be reviewed. The individuals sampled from a single population of *H. kochi* were separated in two distinct groups with high node support [group 7 posterior probability (PP): 100, bootstrap (BS): 85; group 13 PP: 86, BS: 63], probably constituting two sympatric species. Moreover, this result is supported by the high value of intrapopulation sequence divergence (8.6%) of *H. kochi.* The North American species *H. azteca* (subgenus *Hyalella*) was grouped with the species from Minas Gerais state (MG) (subgenus *Mesohyalella*) in a well-supported monophyletic basal clade (PP: 100, BS: 100).

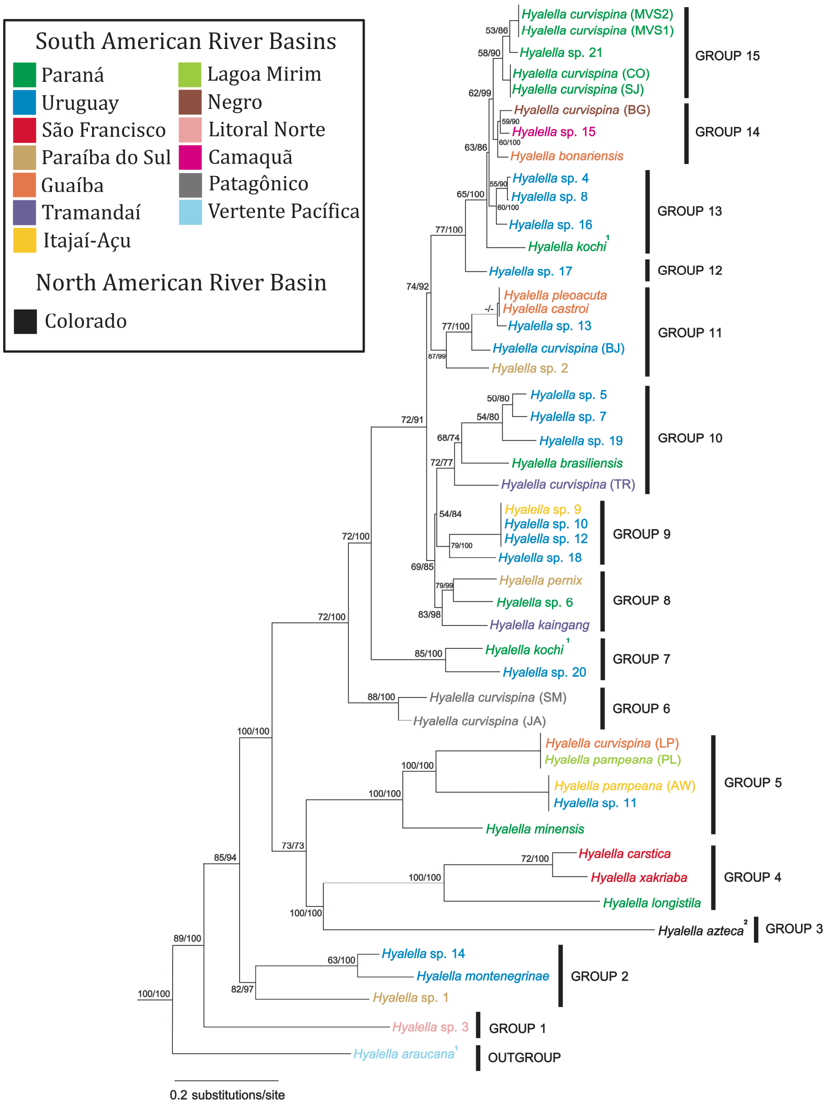

Figure 4 Phylogeny of *Hyalella* reconstructed by Bayesian analysis (BA). Shown is the consensus tree based on 1,513 bp from 16S, 12S and H3 concatenated dataset. Maximum likelihood (ML) analysis resulted in the same topology. ML bootstrap values and BA posterior probabilities are noted above or below the branches (ML/BA in percentages). Values < 50% are not shown. Species are colored according to the River Basin which they occur. Species written without superscript belong to the subgenus *Mesohyalella*; superscript^1 = subgenus *Austrohyalella*; superscript^2 = subgenus *Hyalella*.

As we have included in this study the population of *H. curvispina* from the type locality (Montevideo site 1), as described by Shoemaker (1942), this will be the reference for comparison to the other populations identified as "*H. curvispina*". All the populations of *H. curvispina* from Uruguay were grouped in a monophyletic clade (group 15) with a high node support only for BA (PP: 90). The low values of genetic sequence divergence (values lower than 2.9%) among the populations of *H. curvispina* from Uruguay indicate that they are the same species. Despite the morphological identification have considered the population of San José/Uruguay as a new species (*Hyalella* sp. 21), it did not present differences in the nucleotide sequences (values < 3.3%) among the other populations of *H. curvispina* from Uruguay. Shoemaker (1942), when described *H. curvispina*, stated that the species also occurred in Tramandaí, Rio Grande do Sul state (RS), Brazil. The population of Tramandaí was included in this study and was not grouped with the clade of the populations from Uruguay (Figure 4), indicating that is a distinct species (SST > 12%). Similarly, all populations of "*H. curvispina*" from Brazil and Argentina were polyphyletic (Figure 4), confirming the existence of the species complex "*H. curvispina*".

The two populations of *H. pampeana* were grouped in a monophyletic group with high node support (group 5, PP: 100, BS: 100). We are going to consider the population from Pelotas/RS as the reference for comparison, according to Bueno, Rodrigues and Araujo (2014). Both populations from Pelotas and Alfredo Wagner, Santa Catarina state (SC), did not present differences in the nucleotide sequences (1.6%) from *H. curvispina* of Lagoa do Passo/RS and *Hyalella* sp. 11, respectively. Moreover, other provisional species (*Hyalella* sp. 9, 10 and 12) were grouped in phylogram as a single species, with a high node support (group 9, PP: 79, BS: 100) and low genetic divergence (values < 0.7%).

The sympatric species *H. pleoacuta* and *H. castroi,* in addition to have the lowest value of interpopulation sequence divergence of this work (0.1%), they were grouped in a monophyletic clade (group 11, Figure 4), along with *Hyalella* sp. 13, but with a low value of node support (<50%). Differently, the also sympatric and provisional species *Hyalella* sp. 10 and *Hyalella* sp. 11 presented a high interpopulation sequence divergence (21.6%) and they were separated into distinct groups in phylogram (Figure 4), probably constituting different species.

The divergence dating analysis revealed a late Eocene origin for *Hyalella* with subsequent diversification of extant species occurring in Oligocene and Miocene (Figure 5). The oldest divergence event was the split between *H. araucana* and the other species, at 40.5 million of years ago (mya) on Eocene. The Northern species (those from Minas Gerais state and USA) have been diversified from the remaining species around 33.46 mya. The youngest diversification events were the separation of the populations from Uruguay and some clades that were considered single species by the SST and the phylogram, about 15 and 10 mya, respectively.

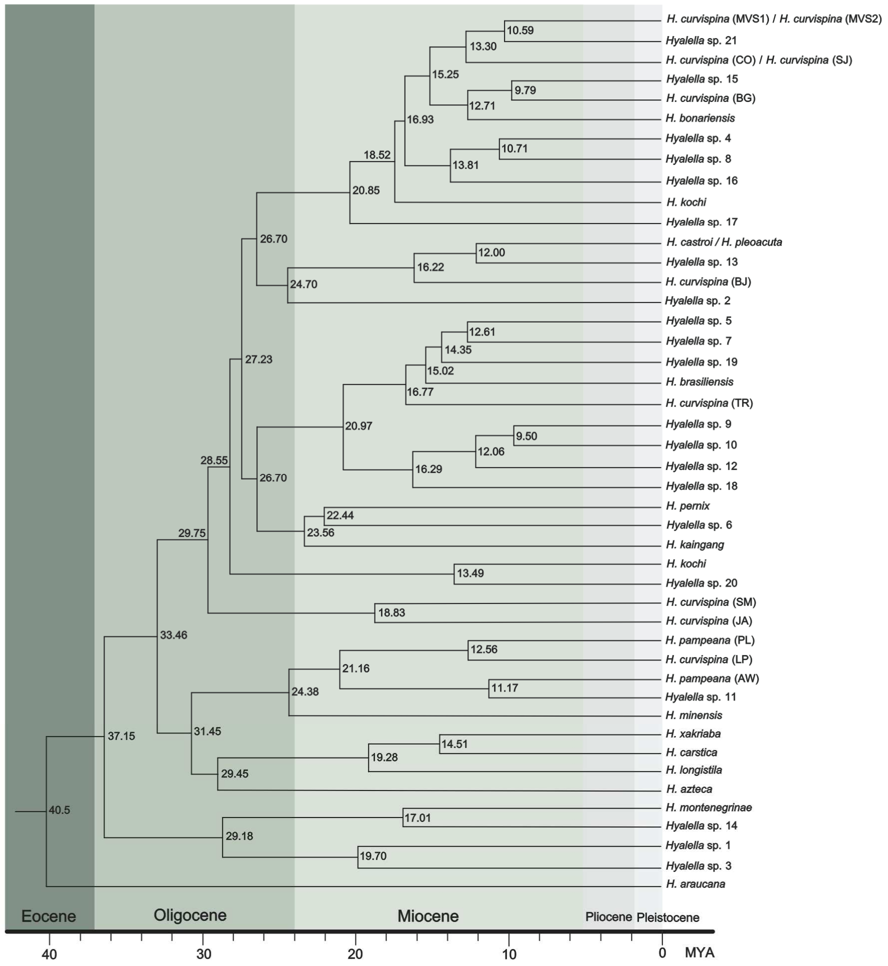

Figure 5 Divergence time chronogram for *Hyalella* species estimated using a Bayesian topology. Divergence time estimates (MYA) are noted adjacent or below to their respective nodes. Geological periods are superimposed onto the phylogeny.

4 DISCUSSION

The application of the species-screening threshold (SST) based on genes intrapopulation divergences is an extremely easy method and can reveal many overlooked species (WITT; THRELOFF; HEBERT, 2006). Several studies have employed the SST as a species recognition method, especially in species complex of invertebrates, including amphipods (WITT; THRELOFF; HEBERT, 2006; KORNOBIS et al., 2010; SKORACKA; DABERT, 2010; BAIRD; MILLER; STARK, 2011).

Although there are objections to the utilization of this tool for it not applied an explicit concept of species, its approach provides a valuable element to the recognition of provisional species that lack in the restricted employment of phylogenetic analysis (HEBERT et al., 2004). Some provisional species identified in this study (e.g. *Hyalella* sp. 15 and *Hyalella* sp. 21) with well-supported clusters could be recognized as different species under the phylogenetic analysis, but not here because their divergences are below the SST. Furthermore, it should be emphasized that DNA barcoding and the SST approach seek to recognize described species and to provisionally recognize undescribed ones, not to define them (WITT; THRELOFF; HEBERT, 2006).

Thus, according to the employment of the SST and the phylogenetic analyzes, we obtained a strikingly number of 20 new species for the genus *Hyalella* in South America, 18 for Brazil and two for Argentina. In this number are included some provisional species and those that were erroneously considered as described species by morphology, such as the populations of *H. curvispina* from Argentina, Tramandaí/RS, Bom Jardim da Serra and *H. pampeana* from Alfredo Wagner/SC. Considering the results obtained here, the species diversity for the genus increased in 29%, 38% to South America and 87% to Brazil (RODRIGUES; BUENO; FERREIRA, 2014; SOUCEK et al., 2015). Some of the provisional species found in this work are already in description process.

Despite the surprising increase in the genus diversity, this result is not unexpected, as apparently the high endemism seems to be common in *Hyalella*. The species complex "*H. azteca*" from North American Great Basin presented similar patterns to the South American species, as an extraordinarily high endemism, the high average value of pairwise nucleotide sequence divergences (around 20%) and the low average intrapopulation divergence (around 4%) (WITT; THRELOFF; HEBERT, 2006, 2008). We have to consider that most of amphipod populations in this study (and also from Great Basin) are from small lakes and springs, with population sizes greatly reduced. Moreover, most of amphipod species present poor dispersal abilities, with subsequent isolation of the populations over time (VÄINÖLÄ et al., 2008). Thus, small effective populations sizes can accelerated rates of DNA sequence evolution, increasing the probabilities that slightly deleterious mutations be fixed by genetic drift, instead of being eliminated (OHTA, 1972; JOHNSON; SEGER, 2001; WOOLFIT; BRORNHAM, 2003).

The species of the subgenus *Austrohyalella* were not monophyletic in the phylogram (Figure 4), and apparently the population of *H. kochi* used in this work consists of two distinct cryptic species, as the morphology could not distinguish them. As mentioned before, Bousfield (1996) created the three subgenus of *Hyalella* based only in morphological analysis (using too subjective appendages body measures) and their geographic distribution. Due to the presence of cryptic species and species complex, and for the species of the subgenus *Austrohyalella* have been paraphyletic in this work, we recommend that this subgenus be invalidated.

The species that occur further North in Brazil formed a monophyletic clade with *H. azteca*, the species from Texas, USA (Figure 4). This result suggests that the migration of the genus may have actually happened from South America to North America, as Bousfield (1996) proposed. Besides that, Witt, Threloff and Hebert (2006) state that *Hyalella* colonized North America no more that 11 million years ago and our results suggest that the origin of the genus occurred at least 40 million years ago. The subgenus *Austrohyalella* was not monophyletic and its validity its refuted, so the species which comprised it may not be the most basal within the genus and not even *Hyalella* has its origin in the South of South America, as Bousfield (1996) stated.

As our analyzes lack an outgroup outside *Hyalella* (which may possibly underestimated and changed the most basal clades of phylogram) and one of the oldest split events was the divergence of the species from MG state and *H. azteca* (Figure 5), we here proposed the hypothesis that *Hyalella* may have its origin in the Northeast of South America. From the appearance of *Hyalella*, possibly a mesohyalellid ancestor, *Hyalella* should have dispersed to Southern South America and North America simultaneously. The fact that other freshwater amphipod families in Brazil only occur mostly in Northeastern (FIŠER; ZAGMAJSTER; FERREIRA, 2013) and to the species of *Mesohyalella* be morphologically similar to amphipod families from Africa, New Zealand and Australia (LOWRY; MYERS, 2013), we believe that this subgenus is the most basal, originated from a marine ancestor. To confirm this new hypothesis, individuals from close amphipod families (as Ceinidae and Chiltoniiidae), as well as species of *Hyalella* from North and Central Americas, Southern South America and East of the Andes Mountains should be included in new phylogenetic molecular approaches.

Most part of the 15 groups formed in the phylogram did not correspond to the actual distribution of the species, as for the river basin as the geographic distance. The distribution of *Hyalella* is difficult to explain using only vicariant events and apparently populations should disperse, both actively and passively through waterfowls and aquatic mammals (PECK, 1975; SWANSON, 1984). Similarly, lineages within a river basin in North America are polyphyletic and their distribution do not correspond to the local biogeographic history (THOMAS; BLINN; KEIM, 1994, 1998; HOGG et al., 1998; DUAN et al., 2000; WITT; THRELOFF; HEBERT, 2006, 2008). Species which occur geographically distant have been grouped in phylogram suggests that the molecular markers used in this study may not have been ideal, and did not show the true evolutionary relationships within *Hyalella*. Nevertheless, our phylogenetic analyzes resulted in trees with identical topologies for the three genes used, indicating that our results are probably correct.

The morphological characteristics used in this work to identify the described and the provisional species were efficient, and could also be applied to populations in North America considered "impossible to be distinguish" (HOGG et al., 1998; WITT; HEBERT, 2000; WELLBORN; COTHRAN, 2004; WITT; THRELOFF; HEBERT, 2006, 2008). However, these characteristics should be used with caution, as they were incapable to distinguish populations of the species complex "*H. curvispina*" and other cryptic species in this work. Furthermore, the morphology did not reflect the actual evolutionary relationships within *Hyalella* (manuscript being prepared), and should be used only for species identification and taxonomy, not to reconstruct the phylogeny of the genus in future researches.

The fact that apparently a single species (or provisional species) presents distinct morphologies in different populations (as in *Hyalella* sp. 9, *Hyalella* sp. 10 and *Hyalella* sp. 12 or *H. pampeana* and *Hyalella* sp. 11) with low genetic divergence may be configure cases of phenotypic plasticity. The ability of a single genotype produce multiple phenotypes is commonplace, and is a result to variation in the environment (PFENNIG et al., 2010). Distinct phenotypes within a single species allow its populations which experience environmental variations survive over the time, avoid extinction and transmit these adaptations to future generations, without necessarily constitute a new species (PFENNIG et al., 2010). Nevertheless, these species (or provisional species) of *Hyalella* can configure different taxa, but the molecular markers used in this study were not able to show the genetic differences among them. New molecular and ecological analyzes should be performed in order to confirm if these are true cases of phenotypic plasticity.

Populations of *H. curvispina* from Argentina and Brazil have shown to be paraphyletic in relation to the population from the type locality in Uruguay (MVS1). Although these populations have been carefully analyzed for their morphology, they showed no apparent differences, constituting cryptic species and confirming the existence of the species complex "*H. curvispina*". Shoemaker (1942) stated that *H. curvispina* occurs in Uruguay and Tramandaí/RS, and through our morphological analysis we verified that they really do not differ from each other. However, the population from Tramandaí was paraphyletic to the populations of Uruguay, constituting a new species and refuting the proposal of Shoemaker (1942).

Despite *H. bonariensis* have formed a monophyletic group and presented values of pairwise nucleotide sequence divergences lower than SST regarding *H. curvispina* from Uruguai, Bagé/RS and *Hyalella* sp. 15, we believe that it is a distinct species. The population of *H. bonariensis* used in this study occurs in a very small spring isolated from other water bodies (article submitted), as well as being very distinct in the morphology from *H. curvispina*. Furthermore, the values of divergence among these species were too close to the SST value, suggesting that they may be different species. Unfortunately, the molecular analysis of the paratypes of *H. bonariensis* were not successful due to the fact as they were fixed in formalin. Thus, while molecular analyzes are not carried out including the population from the type locality, we will consider the population of Silveira Martins as *H. bonariensis*.

In the same way, the population from the type locality of *H. pampeana* was not included in this study, so it is not possible to state that the population from Pelotas/RS, used here as reference (BUENO; RODRIGUES; ARAUJO, 2014) it is actually the same species described by Cavalieri (1968). Therefore, *H. curvispina* from Lagoa do Passo/RS should be consider as *H. pampeana*, as both species were grouped in the phylogram and showed no genetic divergence greater than the SST. On the other hand, the population from Alfredo Wagner/SC considered as *H. pampeana* constitute a new species, along with *Hyalella* sp. 11, from Bom Retiro/SC.

In three localities were found sympatric species of *Hyalella*: São José dos Ausentes/RS, Bom Retiro/SC and Jujuy Province/Argentina. Apparently the existence of sympatric species within the genus is common and may be due to the small populations size, low dispersal ability, physical isolation of their habitats, accelerated rates of DNA sequence evolution and availability of niche diversity in freshwater ecosystems (WELLBORN, 1994; GONZÁLEZ; WATLING, 2003; WELLBORN; COTHRAN, 2004, 2007; CASTIGLIONI; BOND-BUCKUP, 2008a; WITT; THRELOFF; HEBERT, 2008). In addition, the recurrent sympatry in *Hyalella* suggests that each population when sampled should have the morphology analyzed using several individuals, ensuring that ecological, ecotoxicological and distributional studies are not addressing more than one species.

The sympatric species *H. pleoacuta* and *H. castroi* have shown a peculiar case in this work. The two species are very different in morphology: distinct body sizes; number, type and arrangement of setae in appendages; shape and size of gnathopods; and especially the presence of dorsal flanges on pereions and pleons of *H. pleoacuta* (GONZÁLEZ; BOND-BUCKUP; ARAUJO, 2006).

Moreover, it is well studied that the two species present different life strategies, as distinct reproductive periods, use of the habitat (one species inhabits the bottom substrate and the other inhabits closer to the surface), fecundity rates, longevity, among many other aspects (CASTIGLIONI et al., 2007; CASTIGLIONI; BOND-BUCKUP, 2008a, 2008b, 2009). Laboratory cultivation experiments showed that they are reproductively isolated and there is no gene flow among them (CASTIGLIONI et al., 2007). However, the two species presented only 0.1% of genetic divergence in the gene fragments sequenced, and according to the results of the SST and the phylogenetic analysis, they constitute the same species.

It is still difficult to empirically prove cases of sympatric speciation, mainly due to the exclusive use of mitochondrial DNA in phylogenetic analyzes, small sample sizes or use of insufficient outgroups (BARLUENGA et al., 2006; BOLNICK; FITZPATRICK, 2007). It is likely that these two species of *Hyalella* has undergone a process of ecological speciation, which usually refers to speciation driven by ecologically-based divergent selection, causing adaptive divergence and consequent reproductive isolation (GRAVILETS et al., 2007; THIBERT-PLANTE; HENDRY, 2010). Ecological speciation arises as a consequence of the interaction of individuals with abiotic and biotic environment during resource acquisition, with a few examples reported so far (GRAVILETS et al., 2007). A classic example are cichlid fish from Lake Apoyo, Nicaragua, where two *Amphilophus* species strongly differ in eco-morphology traits and resource utilization, but surprisingly with no genetic divergence among them (BARLUENGA et al., 2006). Another well-supported example of ecological speciation are three stickleback fish sympatric species, which they differ in eco-morphology, but also presenting low genetic divergence (TAYLOR; McPHAIL; SCHLUTER, 1997).

The absence or low genetic divergence among sympatric species could be due to the markers used in the analyzes, which may not correspond to the actual differences between the species (TAYLOR; McPHAIL; SCHLUTER, 1997; VIA; WEST, 2008). In addition, when the speciation occurs in a short period of time, the genetic differences might not be visible in the DNA sequences analyzed (JOHANNESSON, 2001; SCHLUTER, 2001). Thibert-Plante and Hendry (2010) also state that when the morphological differences between sympatric species are too discrepant (such as *H. castroi* and *H. pleoacuta*), the genetic differences are much lower among them when compare to sympatric species with few morphological differences (THIBERT-PLANTE; HENDRY, 2010). More molecular analyzes using other markers are necessary to confirm that *H. castroi* and *H. pleoacuta* are really distinct species. However, due to the large eco-morphological traits differences between them, we will consider here that they are different species and this is a probably a case of ecological speciation.

Using the SST, *Hyalella* sp. 13 did not differ from *H. castroi* and *H. pleoacuta* and formed a monophyletic group with both species. However, *Hyalella* sp. 13 has unique morphological features, not only regarding to *H. castroi* and *H. pleoacuta*, but also among the other species of the genus (article in preparation). In this way, considering the peculiar case of ecological speciation mentioned above, we will consider *Hyalella* sp. 13 as a new species, however, ecological studies and laboratory cultivation should be performed in order to confirm this result.

The results obtained in this study have important implications for freshwater ecosystems conservation in South America, specially in Brazil. Habitats where the species of *Hyalella* are found in Brazil are, in general, small lakes, springs, wetlands and small dams (BUENO; RODRIGUES; ARAUJO, 2014). In Brazil, the aquatic invertebrates are the most neglected animals in the Red List of Threatened species. Although they constitute a large part of the country's biodiversity, these organisms do not even have estimates of their threat level, and most of Brazilian conservation projects include only vertebrates and terrestrial ecosystems (ABELL, 2002; MINISTÉRIO DO MEIO AMBIENTE, 2008). Considering the high level of endemism of the species of *Hyalella*, its great diversity and its potential as bioindicators organisms of environmental quality (SOUCEK et al., 2013; WESTON et al., 2013), understand and preserve the diversity of the genus, its ecology and distribution become essential for the conservation of freshwater environments in Brazil and South America.

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ARTIGO 2

Population structure of four freshwater amphipods from different latitudes and biomes in Brazil

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ABSTRACT

Population structures of four freshwater amphipods from Brazil were studied. *Hyalella xakriaba* and *H. longistila*, from lower latitudes, *H. bonariensis* and a new species, here called *Hyalella* sp., from higher latitudes. We tested the hypothesis that life history patterns is related with latitudes. Populations were sampled during the rainy and dry seasons of 2012. The highest abundance of the populations occurred in the winter, which had greater food availability. The reproduction of the species from lower latitudes is continuous throughout the year, and in higher latitudes the reproduction is apparently seasonal. Operational sex ratio favored males in both seasons on species of low latitudes and for the other amphipods, females were more abundant in the summer. Body size appears to be related to the type of habitat and climate, and amphipods from higher latitudes reached larger body sizes. Males presented larger body sizes than females in all species, but they were not significantly larger than ovigerous females. Species that occur closer from the tropics reached sexual maturity at smaller body sizes than species from higher latitudes. Fecundity did not follow the classical patterns of other amphipods, and apparently it is more related to the body size of females than to climate and latitude. Explanations on the life history aspects presented by these amphipods are discussed. The results supported our hypothesis, however, the regional climate and habitat may also influence the life history patterns of freshwater amphipods from the Southern Hemisphere. This paper can contribute to the creation of conservation programs, since freshwater amphipods are endemics and bioindicators of water quality.

Keywords: Abundance; Body size; Fecundity; *Hyalella*; Sex ratio; South America.

1. Introduction

Freshwater ecosystems are extremely important for the survival of a large portion of the organisms and are part of all human activities. Although, around the world, freshwater habitats are subjected to many disturbances caused by humans and nowadays they are considered the most threatened environments in the planet (Abell, 2002).

Studies on the conservation biology of freshwater environments represent only 4% of the articles published on specialized journals (Abell, 2002). Aggravating the scenario, extinction rates predictions for freshwater fauna are five times higher than those of terrestrial and marine animals (Nel et al., 2009; Saunders et al., 2002). The situation is even worse for freshwater invertebrates as there are no estimates of their current threatening risk, despite they constitute a large part of the biodiversity (Dudgeon et al., 2006).

One of the methods that guide the creation of conservation programs is the study of structure and population dynamics, which enables to understand and estimate the populations' size, their stability, reproductive period, immigration and migration rates, the environmental factors that affect them, among many other aspects (Brawn and Robinson, 1996). Some organisms present characteristics that make them ideal for studying the structure and population dynamics with conservation purposes, such as the bioindicators of environmental quality, that may have naturally small populations and are usually unable to disperse (Abell, 2002). Furthermore, endemic species should also be included in conservation studies, because they can easily be extinct by an environmental imbalance (Nel et al., 2009; Suski and Cooke, 2007).

Species of freshwater amphipod crustaceans are commonly endemic and bioindicators of water quality, being widely studied in the Northern Hemisphere (Barnard and Barnard, 1982; Ding et al., 2011). Despite the diversity of amphipods in the Southern Hemisphere is low, regions such as South America certainly has an underestimated number of species, as especially in Brazil new taxa have been discovered in the last few years (Fišer et al., 2013; Rodrigues et al., 2014; Väinölä et al., 2008).

Despite having the majority of freshwater reserves in the world, Brazil has experienced serious water crises due to an inefficient and outdated management of this resource. Thus, the study of freshwater environments in this country and the creation of conservation areas that encompass these ecosystems are urgent (Krol et al., 2001; Lemos and Oliveira, 2004). In addition, Brazil has the highest world diversity of freshwater amphipods from Hyalellidae family, presenting a great potential for studies on their structure and population dynamics, still scarcely known, as well as other aspects of its ecology (Bueno et al., 2014).

In this way, the aim of this paper is to understand the population structure of four freshwater amphipods in Brazil. We tested the hypothesis that freshwater amphipod species from low latitudes present similar life history patterns, and species from high latitudes in Brazil present similar patterns and comparable to the Northern Hemisphere amphipods. These results will assist in the creation of specific conservation programs of freshwater environments in Brazil.

2. Material and methods

2.1 Collection and laboratory analysis

Four species of freshwater amphipods of the genus *Hyalella* (Hyalellidae) were used to perform this work. This genus was chosen because it is the only epigean taxon of freshwater amphipods in South America, whereas the other genera and families are confined to subterranean environments

(Rodrigues et al., 2014). The chosen species were *H. xakriaba* Bueno & Araujo 2013, *H. longistila* (Faxon, 1876), *H. bonariensis* Bond-Buckup, Araujo & Santos, 2008, and a new species that is in description process, which here will be called *Hyalella* sp. These species occur at different latitudes and biomes of Brazil, and distinct types of water bodies (Figure 1).

Hyalella xakriaba occurs in Peruaçu River, a region of palm swamp vegetation at the Environmental Protected Area Cavernas do Peruaçu (14°58'36.5" S; 44°26'36" W), extreme North of the state of Minas Gerais (MG), Southeastern Brazil. This site is considered an ecotone, as it is in the transition between Cerrado and Caatinga biomes. The population is located at 721 meters above the sea level, and occurs in the most preserved habitat among the other species studied.

The population of *H. longistila* was sampled in a stream in a private property in the municipality of Ijaci/MG (21°10'24" S; 44°56'24.2" W), at 903 m of altitude, in a transition area between Cerrado and Atlantic Rain Forest. The stream is used to irrigate a maize plantation and as water source for the cattle, resulting in deterioration and soil compaction of the stream margins, removal of the marginal vegetation.

Hyalella sp. occurs in a small branch of Pessegueiro River, in a private property (27°45'45.6" S; 50°01'49.4" W), in the municipality of Bocaina do Sul, state of Santa Catarina (SC), at 838 m above the sea level. The waste of a stockyard is discharged into this branch, resulting in high amounts of organic matter and macrophytes in the water. The sampling site is located in southern Brazil, in the Atlantic Rain Forest.

Hyalella bonariensis occurs in the municipality of Silveira Martins (29°39'23.5" S; 53°37'37.3" W), in the state of Rio Grande do Sul (RS), southern end of Brazil. This population is found in a small spring of a private property, at

127 m of altitude. The spring is well preserved, and form a very small pool, located in a transition area between the Atlantic Rain Forest and Pampa biomes.

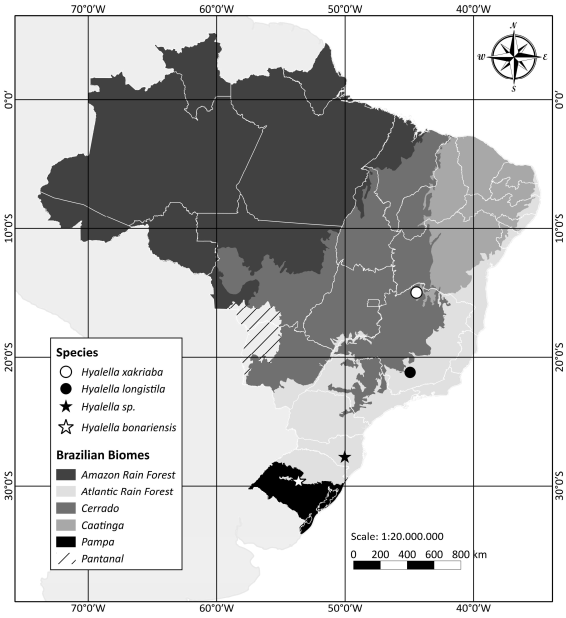

Figure 1 Map of Brazil and its biomes showing the location of the *Hyalella* species studied in this work.

All populations were sampled twice, once on March at the end of the rainy season (summer) of 2012, and another at the end of the dry season (winter), on September of 2012. The amphipods were collected using a small hand net, with sampling effort of two people for 20 minutes. In the field, ovigerous females were identified by naked eye and individualized in microtubes, so that at the time of the preservation in ethanol the eggs were not lost if they released the marsupium. Couples in precopulatory behavior were also individualized, so that their sizes could be compared with the other individuals later. All specimens were preserved in 70% ethanol.

In the laboratory, all animals had the cephalothorax length (CL) measured using a stereoscopic microscope with a micrometric lens. Specimens were classified into four categories: males (individuals with the second pair of gnathopods larger than the first pair); females without eggs in marsupium, which will be considered here only as "females" (individuals with the second pair of gnathopods the same size as the first pair); ovigerous females (females carrying eggs inside the marsupium) and juveniles (Borowsky, 1991; Castiglioni and Bond-Buckup, 2008). We considered as juveniles the individuals who did not have secondary sexual characteristics as well as with a size smaller than the smallest female found in precopulatory behavior. We used this criterion because, unlike males that are easily identified, juveniles and females have similar appearance.

2.2 Data analysis

Normality of the frequency distributions was analyzed using Shapiro-Wilk test (α=0.05). For each population, in both seasons, the total frequency distribution in size classes was estimated regarding the CL of all categories (in this case, ovigerous females were included with females). The width of the size classes was determined using the value of 1/4 of the standard deviation of CL mean values.

For all species the CL values of all categories and the number of eggs of the ovigerous females were expressed as mean ± standard deviation, and minimum and maximum length of CL were also determined. Mean CL sizes were compared using Mann-Whitney test (U; α=0.05), as follows: a) among categories within each species, for both seasons; b) among the same category of the same species, but among seasons; and c) comparing the same category among species, in both seasons.

For each species the operational sex ratio (OSR) was calculated per season. This ratio was estimated as the total number of males divided by the total number of non-ovigerous females, and it was calculated in this way because it is considered only the proportion of males and females that are able to reproduce in a given moment. As ovigerous females are not available to reproduce, they are excluded from the calculation (Emlen, 1976). A Chi-Square test was performed afterwards (χ^2, α=0.05) in order to check if the sex ratio follows the pattern 1 male: 1 female.

In all species, the size that individuals reach sexual maturity was estimated based on the size of the smallest female and the smallest male found forming couples in precopulatory behavior (Castiglioni and Bond-Buckup, 2008). Physico-chemical variables (temperature, dissolved oxygen, pH and electrical conductivity) were recorded in both seasons for all populations.

3. Results

A total of 767 individuals were collected. *Hyalella longistila* was the most abundant species, with 318 specimens. The population of *Hyalella* sp. had 221 individuals sampled, *H. xakriaba* had 109 individuals and the species less abundant was *H. bonariensis*, with only 89 specimens. Table 1 shows the number of males, females, ovigerous females (with the mean number of eggs) and juveniles sampled in each of the seasons for each species.

Apparently, there was a greater recruitment of juveniles during the winter in *H. longistila* and *H. xakriaba* populations, species that occur at lower latitudes. On the species from higher latitudes, *H. bonariensis* and *Hyalella* sp., adults were always more abundant than juveniles, in both seasons (Figure 2).

Table 1 Number of males; females; ovigerous females (in parenthesis its relative percentage to the total number of females in the season); mean value of eggs per ovigerous female ± standard deviation; juveniles and the total number of individuals sampled of four species of *Hyalella* in two seasons.

Latitude	**Species - Season**	**Males**	**Females**	**Ovigerous Females**	**Eggs**	**Juveniles**	**Total**
Low	***H. xakriaba***						
	Summer	20	11	6(35%)	15±6.7	19	56
	Winter	28	8	0(0%)	-	47	83
	H. longistila						
	Summer	49	35	15(30%)	12.3±2.0	49	148
	Winter	30	13	7(35%)	16.8±2.0	120	170
High	***Hyalella* sp.**						
	Summer	23	30	7(19%)	25±4.0	21	81
	Winter	49	38	17(31%)	30±3.7	36	140
	H. bonariensis						
	Summer	11	34	0(0%)	-	25	70
	Winter	14	5	0(0%)	-	0	19
	Total	**224**	**174**	**52**	**-**	**317**	**767**

In the summer, ovigerous females had the greater CL mean size. Only in *H. bonariensis* ovigerous females were not found, and males were the larger specimens. On the other hand, in the winter, males presented the greatest mean size of CL in *H. bonariensis* and *Hyalella* sp., while for *H. xakriaba* females reached the larger sizes and in *H. longistila* ovigerous females were the larger individuals (Table 2). Furthermore, Table 2 also shows the results of normality test and the respective p values for each category of the four *Hyalella* species in both seasons. Regarding species from lower latitudes, females of *H. longistila* (Figure 3) presented a polymodal frequency distribution in the summer (more than two peaks in the curve) and bimodal in the winter (two peaks in the curve). However, the males presented an unimodal distribution in both seasons. For *H. xakriaba* (Figure 4), the distribution of males was bimodal in both seasons and females showed a normal distribution, also in both seasons.

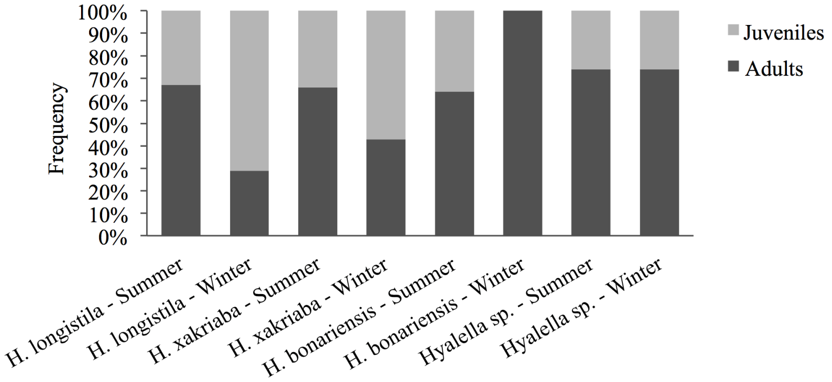

Figure 2 Relative frequency (%) of adults and juveniles of four species of *Hyalella*, in different seasons.

Table 2 Maximum, minimum and mean values of carapace length (mm) ± standard deviation (sd) for different categories of *Hyalella* species, in different seasons of the year.

Species - Category	**Summer**			**Winter**		
	Mean ± sd	**Max.**	**Min.**	**Mean ± sd**	**Max.**	**Min.**
H. xakriaba						
Males	0.53 ± 0.08	0.64	0.42	0.48 ± 0.05	0.58	0.40
Females	0.48 ± 0.07	0.58	0.42	0.51 ± 0.01	0.53	0.49
Ovigerous females	0.58 ± 0.05	0.66	0.52	-	-	-
Juveniles	0.31 ± 0.05	0.38	0.22	0.34 ± 0.05	0.41	0.25
H. longistila						
Males	0.54 ± 0.09	0.75	0.39	0.55 ± 0.09	0.75	0.39
Females	0.47 ± 0.07	0.80	0.39	0.48 ± 0.09	0.75	0.39
Ovigerous females	0.57 ± 0.08	0.80	0.39	0.62 ± 0.06	0.72	0.55
Juveniles	0.32 ± 0.03	0.36	0.18	0.31 ± 0.03	0.36	0.18
***Hyalella* sp.**						
Males	0.63 ± 0.09	0.74	0.40	0.69 ± 0.07	0.86	0.60
Females	0.61 ± 0.06	0.74	0.50	0.61 ± 0.06	0.74	0.50
Ovigerous females	0.67 ± 0.04	0.72	0.62	0.64 ± 0.04	0.70	0.58
Juveniles	0.38 ± 0.06	0.44	0.20	0.35 ± 0.06	0.46	0.25
H. bonariensis						
Males	0.51 ± 0.07	0.60	0.40	0.62 ± 0.06	0.70	0.48
Females	0.46 ± 0.06	0.55	0.35	0.51 ± 0.02	0.53	0.48
Juveniles	0.32 ± 0.02	0.34	0.25	-	-	-

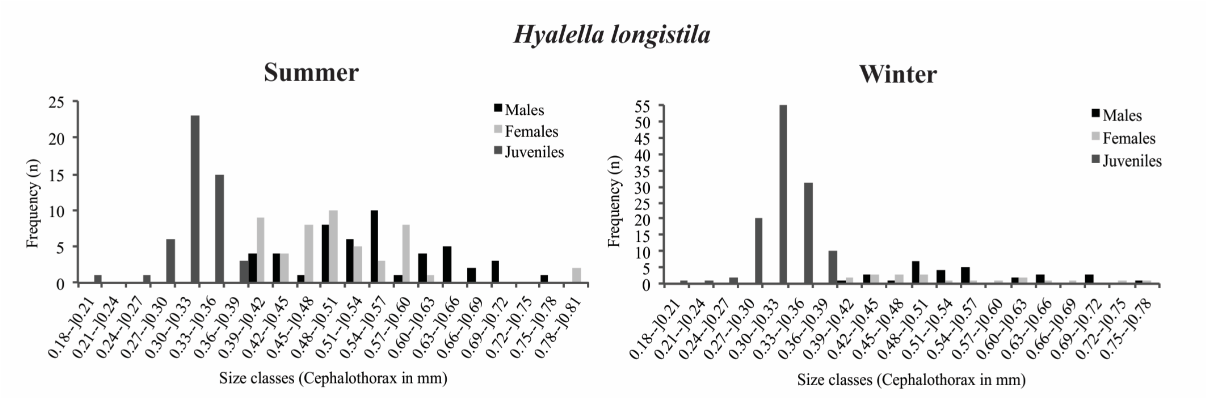

Figure 3 Absolute frequency distribution in size classes of *Hyalella longistila*, in two seasons.

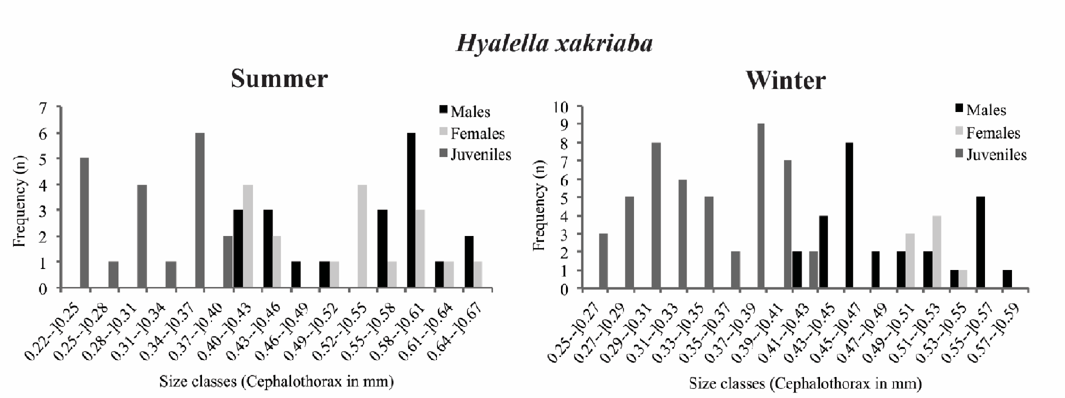

Figure 4 Absolute frequency distribution in size classes of *Hyalella xakriaba*, in two seasons.

The males and juveniles of the species from higher altitudes, *H. bonariensis* (Figure 5) and *Hyalella* sp. (Figure 6), in general, presented a bimodal frequency distribution. The females of both species presented an unimodal frequency distribution in both seasons.

A large proportion of individuals of *Hyalella* sp. were parasitized by Acanthocephala. In the summer, 34% of adults were infected, whereas 39% of males and 30% of females had one or more cystacanths. In the winter, 30% of adults had parasites, 30% of males and 22% of females. In both seasons, parasites were not observed on the populations of the other studied species of *Hyalella*.

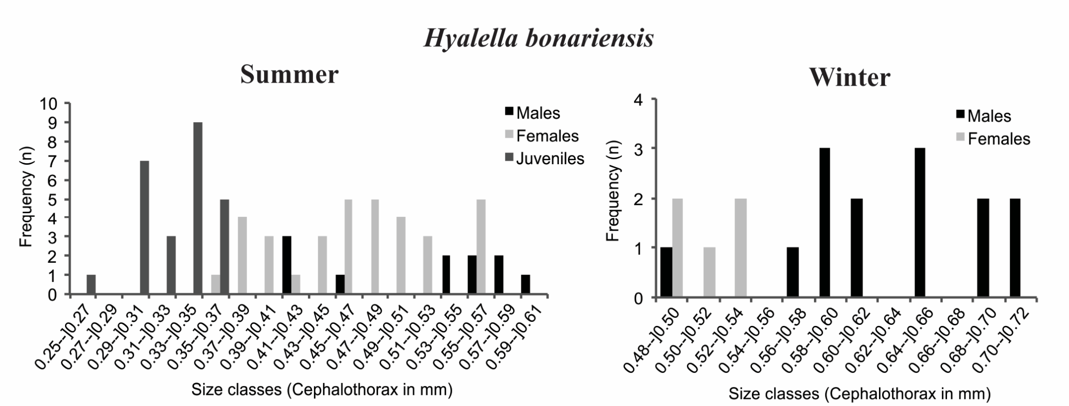

Figure 5 Absolute frequency distribution in size classes of *Hyalella bonariensis*, in two seasons.

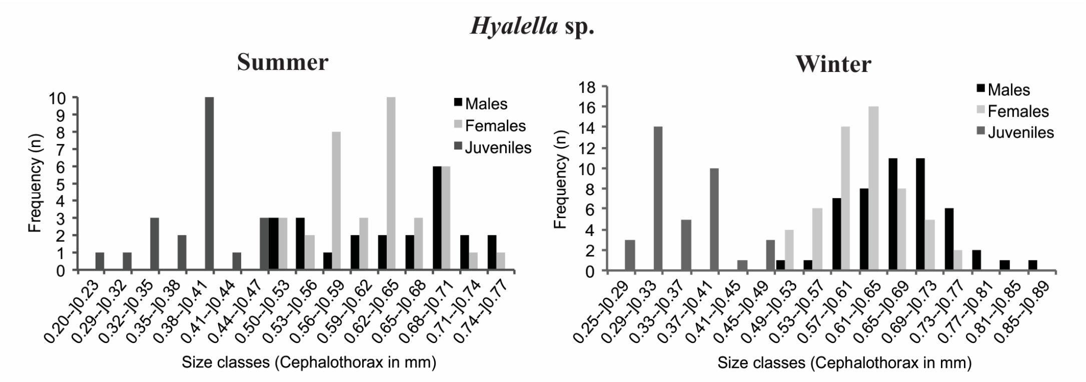

Figure 6 Absolute frequency distribution in size classes of *Hyalella* sp., in two seasons.

In *H. longistila*, ovigerous females were the largest individuals in both seasons, although, they were not significantly different from males in the summer. In both samples, the males were significantly larger than the females, as well as the juveniles were significantly smaller than other categories (Table 3). In the summer, the largest individuals of *H. xakriaba* were ovigerous females, however, they did not present a significant difference when compared to males. In the winter, the largest specimens were females, which also showed no differences when compared to males (Table 4). The males of *H. bonariensis* reached the greatest mean size of CL among the other categories, with significant differences between the size of males and females in both seasons (Table 5). The population of *Hyalella* sp., in the summer, showed no significant differences of mean CL sizes between ovigerous females and males. However, in the winter, the males were significantly larger than ovigerous females, and these did not differ from the females (Table 6).

When the categories of CL mean sizes of *H. longistila* were compared between summer and winter, only ovigerous females presented significant differences among seasons, being larger in the winter (Table 7). Males of *H. xakriaba* were significantly larger in the summer and larger in the winter for *H. bonariensis.* Juveniles of *H. xakriaba* were significantly larger in the winter (Table 7). All categories of *Hyalella* sp. did not present differences on the CL mean sizes among seasons, except juveniles, who were significantly larger in the summer (Table 7).

Table 3 Values of the Mann-Whitney mean test (U) and the respective p values (α=0.05) in parenthesis, comparing the cephalothorax length means (mm) of different categories of the species *Hyalella longistila*, in different seasons of the year. OF=ovigerous females.

Category	**Summer**			**Winter**		
	Females	**OF**	**Juveniles**	**Females**	**OF**	**Juveniles**
Males	375.5(<0.01*)	270.5(0.12)	0(<0.01*)	95.5(<0.01*)	46.5(0.02*)	0(<0.01*)
Females	-	56(<0.01*)	0(<0.01*)	-	7(0.01*)	0(<0.01*)
OF	-	-	0(<0.01*)	-	-	0(<0.01*)

*Indicate significant p value (p<0.05).

Table 4 Values of the Mann-Whitney mean test (U) and the respective p values (α=0.05) in parenthesis, comparing the cephalothorax length means (mm) of different categories of the species *Hyalella xakriaba*, in different seasons of the year. OF=ovigerous females.

Category	**Summer**			**Winter**		
	Females	**OF**	**Juveniles**	**Females**	**OF**	**Juveniles**
Males	64.5(0.06)	42(0.28)	0(<0.001*)	72(0.13)	-	4.5(<0.001*)
Females	-	8(0.01*)	0(<0.001*)	-	-	0(<0.001*)
OF	-	-	0(<0.001*)	-	-	-

*Indicate significant p value (p<0.05).

Table 5 Values of the Mann-Whitney mean test (U) and the respective p values (α=0.05) in parenthesis, comparing the cephalothorax length means (mm) of different categories of the species *Hyalella bonariensis*, in different seasons of the year.

Category	**Summer**		**Winter**	
	Females	**Juveniles**	**Females**	**Juveniles**
Males	107(0.03*)	0(<0.001*)	4.5(0.003*)	-
Females	-	5.5(<0.001*)	-	-

*Indicate significant p value (p<0.05).

Table 6 Values of the Mann-Whitney mean test (U) and the respective p values (α=0.05) in parenthesis, comparing the cephalothorax length means (mm) of different categories of the species *Hyalella* sp., in different seasons of the year. OF=ovigerous females.

Category	**Summer**			**Winter**		
	Females	**OF**	**Juveniles**	**Females**	**OF**	**Juveniles**
Males	261.5(0.13)	65(0.458)	8(<0.001*)	432(<0.001*)	252.5(0.01*)	0(<0.001*)
Females	-	37(0.008*)	0(<0.001*)	-	224.5(0.07)	0(<0.001*)
OF	-	-	0(<0.001*)	-	-	0(<0.001*)

*Indicate significant p value (p<0.05).

Table 7 Values of the Mann-Whitney mean test (U) and the respective p values (α=0.05) in parenthesis, comparing the cephalothorax length means (mm) of the same category among summer and winter samples in four species of *Hyalella*. OF=ovigerous females.

Category	***H. xakriaba***	***H. longistila***	***Hyalella* sp.**	***H. bonariensis***
Males	176.5(0.03*)	721(0.89)	435.5(0.12)	14.5(<0.001*)
Females	33(0.38)	223(0.92)	547(0.77)	44.5(0.09)
OF	-	23.5(0.04*)	29.5(0.06)	-
Juveniles	303.5(0.04*)	2824(0.68)	239.5(0.02*)	-

*Indicate significant p value (p<0.05).

In general, species from higher latitudes presented a greater mean body size than species from lower latitudes. In the summer males of *Hyalella* sp., were significantly larger than those of other species and, in the winter, the body size of all species were different from each other (Table 8). In addition, females of *Hyalella* sp. were also significantly larger than those of other species in the summer and winter (Table 9). Ovigerous females of *Hyalella* sp. were significantly larger in the summer, and in the winter there was no difference on size among species (Table 10). Regarding juveniles, once again individuals of *Hyalella* sp. were significantly larger than others in the summer, and in the winter, despite having reached the greatest mean size, they showed no significant differences from the juveniles of *H. longistila* (Table 11).

Table 8 Values of the Mann-Whitney mean test (U) and the respective p values (α=0.05) in parenthesis, comparing the cephalothorax length means (mm) among males of different species of *Hyalella*, in different seasons of the year.

Species	**Summer**			**Winter**		
	H. xakriaba	***H. bonariensis***	***Hyalella* sp.**	***H. xakriaba***	***H. bonariensis***	***Hyalella* sp.**
H. longistila	482(0.9)	243(0.6)	268(<0.01*)	245(0.006*)	103(<0.01*)	205(<0.01*)
H. xakriaba	-	85(0.3)	99(0.01*)	-	13(<0.01*)	14(<0.01*)
H. bonariensis	-	-	45(0.02*)	-	-	192(0.01*)

*Indicate significant p value (p<0.05).

Table 9 Values of the Mann-Whitney mean test (U) and the respective p values (α=0.05) in parenthesis, comparing the cephalothorax length means (mm) among females of different species of *Hyalella*, in different seasons of the year.

Species	**Summer**			**Winter**		
	H. xakriaba	***H. bonariensis***	***Hyalella* sp.**	***H. xakriaba***	***H. bonariensis***	***Hyalella* sp.**
H. longistila	174.5(0.64)	575(0.81)	48(<0.001*)	19(0.01*)	13(0.06)	43(<0.001*)
H. xakriaba	-	172.5(0.71)	29.5(<0.001*)	-	20(0.93)	14(<0.001*)
H. bonariensis	-	-	35.5(<0.001*)	-	-	9(0.001*)

*Indicate significant p value (p<0.05).

Table 10 Values of the Mann-Whitney mean test (U) and the respective p values (α=0.05) in parenthesis, comparing the cephalothorax length means (mm) among ovigerous females of different species of *Hyalella*, in different seasons of the year.

Species	**Summer**		**Winter**	
	H. xakriaba	***Hyalella* sp.**	***H. xakriaba***	***Hyalella* sp.**
H. longistila	39.5(0.69)	7(0.001*)	-	46(0.405)
H. xakriaba	-	3.5(0.01*)	-	-

*Indicate significant p value (p<0.05).

Table 11 Values of the Mann-Whitney mean test (U) and the respective p values (α=0.05) in parenthesis, comparing the cephalothorax length means (mm) among juveniles of different species of *Hyalella*, in different seasons of the year.

Species	**Summer**			**Winter**		
	H. xakriaba	***H. bonariensis***	***Hyalella* sp.**	***H. xakriaba***	***H. bonariensis***	***Hyalella* sp.**
H. longistila	431.5(0.64)	510(0.23)	144.5(<0.001*)	2180(0.02*)	-	1579(0.01*)
H. xakriaba	-	201(0.38)	62(<0.001*)	-	-	749.5(0.37)
H. bonariensis	-	-	85.5(<0.001*)	-	-	-

*Indicate significant p value (p<0.05).

Males were more abundant than females in both seasons, being favored in sex ratio on the species of low latitudes, *H. longistila* and *H. xakriaba.* For the species from higher latitudes, *H. bonariensis* and *Hyalella* sp., females were more abundant in the summer and males in the winter (Figure 7; Table 12).

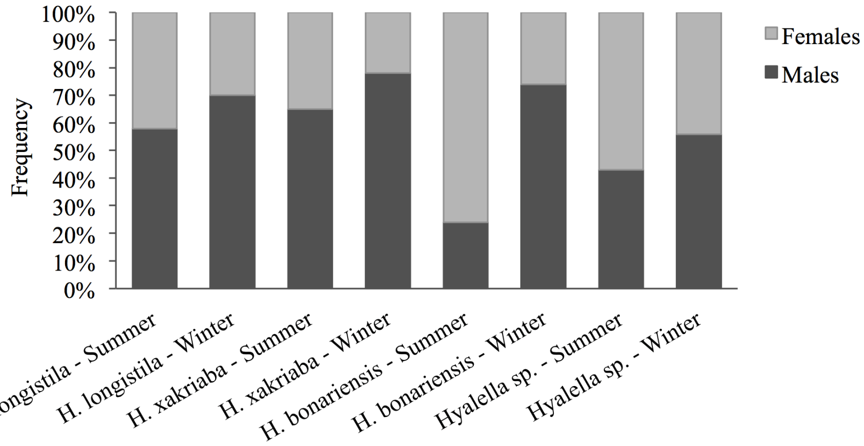

Figure 7 Percentage of males and females of four species of *Hyalella*, sampled in summer and winter of 2012.

Table 12 Proportion among males and females and the values of the Chi-Square test (χ^2) and the respective p values (α=0.05), comparing the operational sex ratio of four populations of different species of *Hyalella*, in two seasons of the year. M=males; F=females; SM=sexual maturity (cephalothorax length in mm).

Species - Season	**M:F**	**χ^2 (p value)**	**SM males**	**SM females**
H. xakriaba				
Summer	1: 0.55	2.613(0.106)	0.5	0.47
Winter	1: 0.28	11.111(<0.001*)		
H. longistila				
Summer	1: 0.71	2.333(0.126)	0.42	0.4
Winter	1: 0.43	6.721(0.009*)		
***Hyalella* sp.**				
Summer	1: 1.3	0.925(0.33)	0.6	0.52
Winter	1: 0.77	1.391(0.23)		
H. bonariensis				
Summer	1: 3.09	11.756(<0.001*)	0.4	0.38
Winter	1: 0.35	4.263(0.03*)		

*Indicate significant p value (p<0.05).

Sexual maturity in all species was reached in larger sizes of CL by males, when compared to females. *Hyalella* sp. was the species that had the larger body size at sexually maturity and *H. bonariensis* had the smaller body sizes at sexual maturity (Table 12).

The values of the physico-chemical variables are shown on Table 13.

Table 13 Values of the physico-chemical variables of water measured in two seasons of the year in four populations of different species of *Hyalella* in Brazil. DO=dissolved oxygen; EC= electrical conductivity; Temp=temperature.

Species - Season	DO (ppm)	pH	EC (µS/cm)	Temp (°C)
H. xakriaba				
Summer	5.3	7.3	120	26.7
Winter	4.92	7.4	124	24.2
H. longistila				
Summer	6.4	5.4	103	24.3
Winter	2.68	7.54	113	24.5
***Hyalella* sp.**				
Summer	1.3	7.0	12.5	25
Winter	1.2	7.0	13	23
H. bonariensis				
Summer	4.71	6.9	51	23.1
Winter	4.66	7.2	53	22.4

4. Discussion

4.1 Population structure

Studied populations were more abundant in the winter, except *H. bonariensis.* This pattern differs from the Northern Hemisphere amphipods, which are more abundant in the summer, as *H. azteca* (Cooper, 1965; Hogg et al., 1995; Pilgrim and Burt, 1993; Strong, 1972), *H. montezuma* (Dehdashti and

Blinn, 1991), *Gammarus palustris* (Van Dolah, 1978), *G. salinus* (Skadsheim, 1989) and *G. pulex* (Graça et al., 1994; Kelly et al., 2006; Mortensen, 1982).

In temperate environments, summer and spring temperatures around 25°C are ideal for the reproduction of the amphipods, since with the greater food availability, they perform the molt more often, grow and reach the maturity earlier (Cooper, 1965; Panov and Mcqueen, 1998). In addition, higher temperatures stimulate the production of larger broods and eggs (Siegfried, 1985) and increasing juveniles survival rates (Panov and Mcqueen, 1998; Skadsheim, 1989; Van Dolah, 1978).

However, water temperatures above 30°C can decrease the abundance of *Hyalella* populations, since it affects growth patterns and increases mortality rates of the amphipods (Hogg et al., 1995; Oberlin and Blinn, 1997; Pilgrim and Burt, 1993). Thus, environmental impacts that increase the temperature of aquatic ecosystems, such as global warming, can directly influence the amphipods survival, making species from tropical environments and low latitudes extremely susceptible to extinction (Hogg et al., 1995; Kelly et al., 2006; Pilgrim and Burt, 1993).

Results of this research are similar to the patterns of abundance of freshwater amphipods from the Southern Hemisphere. Along with the absence of a severe winter, the small variation of temperature and other abiotic parameters observed probably did not influence the abundance of *H. xakriaba*, *H. longistila* e *Hyalella* sp. On the other hand, food availability - specially the presence of macrophytes - must be the main limiting factor, as noted in *H. castroi*, *H. pleoacuta* (Castiglioni and Bond-Buckup, 2008), *H. curvispina* (Casset et al., 2001), *H. montezuma* (Dehdashti and Blinn, 1991) and several freshwater amphipods species from Australia (Smith and Williams, 1983). In the rainy season sample we observed that the amount of macrophytes was much lower than in the dry season, and could be a result of the greater water quantity and flow, washing the plants from the sampled sites.

Morino (1978), Wildish (1988) and Sainte-Marie (1991) reported that amphipods from high latitudes may present similar life history patterns, regardless the biome and habitat where they live. This could explain the similarity among *H. bonariensis* and the Northern Hemisphere species, with respect to a greater abundance in the summer. This result could also be due to the peculiar habitat of the species, a spring in the shape of a pool, which does not allow the creation of current. Consequently, the amount of macrophytes is constant, providing a continuous supply of food and shelter in all seasons.

Juveniles of *H. xakriaba* and *H. longistila* were more abundant than adults the winter, and could be the result of low levels of dissolved oxygen, due to the large amount of macrophytes and low water flow. Amphipods from the same population present different respiratory demands, so that larger individuals require even four times more oxygen than smaller individuals (Auel and Ekau, 2009; Gaston and Spicer, 1998). Furthermore, the greater abundance of juveniles in the winter may indicate that the reproduction of these species is synchronized with the period of greatest food availability, a fact that has been widely observed in freshwater amphipods (Casset et al., 2001; Castiglioni & Bond-Buckup, 2009; Nelson, 1980; Smith and Williams, 1983; Strong, 1972; Wellborn, 1995).

However, the fact that ovigerous females of both species were found in summer and winter suggests that reproduction should be continuous throughout the year. Sainte-Marie (1991) states that amphipods from low latitudes and warm climates are iteroparous (reproduce several times during the life) and multivoltines (reproduce more than once a year), with reproduction peaks during periods of favorable climate conditions. These same patterns were observed in *H. xakriaba* and *H. longistila*, as well as other Southern

Hemisphere amphipods, as *H. castroi*, *H. pleoacuta* (Castiglioni and Bond-Buckup, 2008), *H. curvispina* (Casset et al., 2001), *Pseudomoera gabrielli* and *Austrochiltonia australis* (Smith and Williams, 1983).

Adults were more abundant than juveniles in both seasons in the species of high latitudes, *H. bonariensis* and *Hyalella* sp. The absence of ovigerous females of *H. bonariensis* suggests that its reproduction should occur only at a certain period of the year, probably out of the sample of this research. As the habitat of this species is very restricted, the annual reproduction could also avoid the competition among juveniles and adults for food and shelter (Wellborn, 2002, 1994; Wellborn and Bartholf, 2005; Wellborn and Broughton, 2008; Wellborn and Cothran, 2007a). Apparently, *H. bonariensis* shows the same patterns of amphipods from higher latitudes (Cooper, 1965; Kelly et al., 2006; Nelson, 1980; Xinqing et al., 2013) which are generally iteroparous or semelparous (reproduce only once throughout life, dying soon after) and univoltines, reproducing only once per year (Sainte-Marie, 1991).

As ovigerous females were found in both seasons, the reproduction of *Hyalella* sp. should occur throughout the year. Nevertheless, juveniles were not the most abundant, possibly due to the high number of amphipods parasitized by Acanthocephala, resulting in a lower reproduction rate and a lower recruitment. Acanthocephalans utilize amphipods as intermediate hosts to reach the final host, usually a fish or a waterfowl (Kaldonski et al., 2009). To complete their life cycle, parasites reduce the fecundity of female amphipods and may even make them infertile, besides reducing the successful pairing of males (Bollache et al., 2002; Rauque and Semenas, 2009). Parasites also alter the physiology (Plaistow et al., 2001), the behavior and the amphipod coloration, making them more susceptible to predation by fish and waterfowls (Bakker et al., 1997; Baldauf et al., 2007).

4.2 Frequency distributions and operational sex ratio (OSR)

Amphipods, in general, present a bimodal or polymodal frequency distribution in size classes, which may reflect reduced growth rates of juveniles, recruitment pulses, influence of abiotic variables, migration, mortality by predation based on size, among other factors (Cooper, 1965; Munguia et al., 2007; Newman and Waters, 1984; Ward, 1983).

Studied species showed different frequency distributions, so that *H. xakriaba*, *Hyalella* sp. and *H. bonariensis* were similar, being generally bimodal or polymodal. These types of distributions imply that these populations have reproduction peaks, with a continuous input of juveniles throughout the year (Pickard and Benke, 1996). This has been observed in other species, as *H. pleoacuta*, *H. castroi* (Castiglioni and Bond-Buckup, 2008) and *H. curvispina* (Casset et al., 2001).

Despite males of *H. bonariensis* had a bimodal distribution in both seasons, it can not be stated that the population reproduces over the year. The absence of ovigerous females in both seasons and juveniles in the winter suggests that *H. bonariensis* has a seasonal reproduction, typical of amphipods from high latitudes (Nelson, 1980; Sainte-Marie, 1991). Collections of this population for a longer period of time, as well as other seasons, should be performed to confirm these results.

Polymodal distribution of juveniles can be explained by the occurrence of several recruitments over time, reinforcing the hypothesis that reproduction is continuous, usual for species from tropical and warm climates (Sainte-Marie, 1991). Unimodal frequency distribution observed in some categories is unusual and may be the result of the limited number of samples and the small size of the population, so that more samples should be made in order to confirm this pattern.

Along with body size and frequency distribution, the analysis of sex ratio is an important evaluator of the reproductive potential of populations and their long-term survival (Lemaître et al., 2009). In this paper, sex ratio of the species from lower latitudes favored males in both seasons, while for *H. bonariensis* and *Hyalella* sp. females were more abundant in the summer and males in the winter.

Differently from our results, it is common that sex ratio varies seasonally in amphipods, and generally females are more abundant than males, as observed in *Hyalella* species (Casset et al., 2001; Castiglioni and Bond-Buckup, 2008; Dehdashti and Blinn, 1991; Strong, 1972) and other amphipods (Appadoo and Myers, 2004; Ladewig et al., 2006; Powell and Moore, 1991; Smith and Williams, 1983).

Apparently, the reason for sex ratio favors females is males' behavior. There are no known chemical communications in *Hyalella*, so that males can not perceive when females are able to reproduce and they need to explore the environment continuously in order to find a mate (Cothran, 2008; Wellborn, 1995). This make males more susceptible to predation and parasitism, favoring females on sex ratio (Newman and Waters, 1984; Wellborn and Bartholf, 2005). Furthermore, fish and Odonata larvae preferably prey the largest individuals of *Hyalella*, in this case the males, in a way that females become even more abundant (Wellborn, 1994).

However, in this work, the OSR was carried out, and not merely sex ratio. OSR was defined by Emlen (1976) as the potential rate of males and females that are able to reproduce in a given moment, and is accepted as a good reproductive predictor for crustaceans. A male-based OSR is a result of asynchronous reproductive receptiveness of females and to the limited time that they are available to reproduce, for *Hyalella* is a short period after the molt (Emlen and Oring, 1977; Wellborn, 1995). In most animals, the reproductive potential of males is higher than of females, resulting in a male-based OSR (Emlen and Oring, 1977). This pattern was observed in *H. longistila* and *H. xakriaba* in both seasons, but with significant differences in sex ratio only in the winter. Thus, a male-based OSR observed for these two species suggests that their reproduction is continuous, due to asynchrony of females, confirming that amphipods from warm climate and low latitudes reproduce throughout the year (Nelson, 1980; Sainte-Marie, 1991).

Nevertheless, *H. bonariensis* and *Hyalella* sp. presented a male-based OSR only in the winter. The highest percentage of amphipods parasitized by Acanthocephala in the summer in *Hyalella* sp. may have influenced the female-based OSR. Due to the acanthocephalans lifecycle, the parasitism rate is higher in the summer, and as generally larger individuals of amphipods are the most parasitized (males of *Hyalella*), they will have a higher mortality rate, resulting in a female-based OSR (Rauque and Semenas, 2009, 2007; Uznanski and Nickol, 1980).

Moore (1981) states that is common northern amphipods present seasonal variations on sex ratio, usually with dominance of males in colder months and females' dominance in warmer periods, pattern that may have occurred in the species from higher latitudes of our study, *H. bonariensis* and *Hyalella* sp. However, due to the small population size of *H. bonariensis* and the OSR values had not been significantly different in both seasons, more samples are necessary to check if there is a pattern in its sex ratio.

4.3 Body size, sexual maturity and fecundity

Sainte-Marie (1991) e Nelson (1980) assert that the body size of the amphipods presents patterns related mainly to climate and latitude. Compared to warm climates, species from cold climate and high latitudes have greater longevity and a large body size, while species from warm climate and low latitudes have shorter longevity and reach small body sizes.

Hyalella sp. had a CL mean significantly larger in all categories, in both seasons. The species occurs in a municipality that is part of Serra Geral, the largest mountain chain in Brazil, considered the coldest region of the country. The rugged terrain of Serra Geral coupled with the humid subtropical climate facilitates snowfall during the winter, reaching temperatures of -10°C (Behling, 1995). Thus, despite *Hyalella* sp. not be the species that occurs in the highest latitude in our study, it probably presented the largest body sizes due to the cold environment in which lives, following the pattern proposed by Sainte-Marie (1991).

Despite being the species that occurs in the highest latitude, *H. bonariensis* showed no significant differences when compared to the species from lower latitudes, presenting the smallest CL mean in the summer. This may be due to habitat with limited space, which could increase intraspecific competition, where the smaller the more successful the individual will be in resource exploitation and reproduction (Wellborn, 2002, 1995, 1995; Wellborn and Bartholf, 2005; Wellborn and Cothran, 2007a). However, in the winter, the fact that the CL means of adults were higher than in the summer and juveniles have not been found suggests again that *H. bonariensis* has a seasonal reproduction. Based on the frequency of the size classes and the CL values we suggest that reproduction occurs in the summer, as population comprises juveniles and young adults. In the winter the reproduction does not occur, and population comprises older adults, reflected in a greater CL mean.

Body sizes of *H. xakriaba* and *H. longistila*, in general, were not significantly different. In the summer, these species did not exhibit the lowest CL means, however, they were not significantly larger than *H. bonariensis.* On winter, *H. longistila* and *H. xakriaba* showed the smallest CL sizes, indicating that the pattern of Sainte-Marie (1991) and Nelson (1980) for amphipods from low latitudes and warm climate could be observed in our study.

Juveniles of all species obviously were significant smaller than adults, as observed in other amphipods (Casset et al., 2001; Castiglioni and Bond-Buckup, 2008; Dehdashti and Blinn, 1991; Smith and Williams, 1983; Strong, 1972). Males were significantly larger than females in all species in both seasons, except for *H. xakriaba* in the winter, but there was no significant difference. It is well documented for crustaceans that males are larger than females, and this difference is marked for freshwater amphipods (Castiglioni and Bond-Buckup, 2008; Dehdashti and Blinn, 1991; Wellborn, 1995, 1994; Wellborn and Cothran, 2007a).

Low (1978) states that males and females present similar growth rates until they reach maturity, and from that moment they invest different amounts of energy in reproduction and other biological demands, resulting in distinct growth rates. Thus, in amphipods, females grow slower due to the prolonged intermolt period, since they invest more energy in production and incubation of eggs, not performing the molt during this time (Borowsky, 1991; Wellborn and Cothran, 2007b). Males, on the other hand, invest their energy on growth and reproductive effort of pairing with a female, reaching larger body sizes, since they constantly perform the molt (Borowsky, 1991; Wellborn and Cothran, 2007b). Precopulatory behavior of *Hyalella* also favors larger males, since they carry females for days until the copula occurs. In addition, females are more receptive to large males, being these individuals the ones with the highest success pairing rates (Wellborn, 1995; Wellborn and Cothran, 2007b).

Ovigerous females presented mean body sizes larger than males in all species where they were found, however, in most cases these differences were not significant. As one of the few exceptions within amphipods, members of the superfamily Talitroidea (where Hyalellidae is inserted) have species where the maximum body size of females exceeds or equals the males (Sainte-Marie, 1991). It is well established that female size is proportional to the number of eggs she produces, being advantageous for them also invest energy in growth, because they will be more fecund (Borowsky, 1991; Wellborn and Cothran, 2007b). As previously mentioned, males are more exposed on the environment searching for a mate, making them more susceptible to predation. Therefore, larger males will inevitably be the most preyed individuals, making males with an intermediate size more abundant, which could explain the fact that ovigerous females present equal or superior size compared to males (Wellborn, 1994, 1995).

Body size at which individuals reach sexual maturity is also related to latitude and climate. Amphipods from high latitudes and cold climate tend to grow slowly and mature late, as observed in *Hyalella* sp. On the other hand, amphipods from tropics and warm climate grow faster and mature earlier (Sainte-Marie, 1991), as *H. xakriaba* and *H. longistila*. This pattern happens because high temperatures increase growth rates, causing the maturation of the individuals of smaller sizes (Cooper, 1965; Pilgrim and Burt, 1993). Apart from that apparently there is a negative relationship between water temperature and animal size (Panov and Mcqueen, 1998). Another abiotic factor that can affect the growth of amphipods is the electrical conductivity. High conductivity levels can influence distribution, abundance and accelerate the growth of crustaceans, as it provides ideal quantities of ions necessary to perform the molt (Kestrup and Ricciardi, 2010; Meyran, 1997; Rukke, 2002). The high electrical conductivity levels recorded in the occurrence sites of *H. xakriaba* and *H. longistila* may help explain their early maturation.

The only species that does not fit the pattern of sexual maturity proposed by Sainte-Marie (1991) was *H. bonariensis*. As already discussed, the restricted habitat of this species probably limits the population's size, so that this ecological pressure may have selected the smaller individuals in order to avoid the instraspecific competition, allowing its presence in that environment (Wellborn, 2002, 1995, 1994; Wellborn and Bartholf, 2005; Wellborn and Cothran, 2007a).

Finally, Sainte-Marie (1991) and Nelson (1980) state that fecundity of amphipod females from cold climate and high latitudes is much lower when compared to tropical species. This pattern was not observed in our study, where *Hyalella* sp., which occurs in the coldest environment, presented the greatest mean of eggs per female, while the species closest from the tropics showed a lower fecundity. Moreover, *H. castroi* and *H. pleoacuta* that occurs at higher latitudes in Brazil of the species studied in this paper, present a fecundity even greater than *H. longistila, H. xakriaba* and *Hyalella* sp. (Castiglioni and Bond-Buckup, 2009), not following the pattern of Sainte-Marie (1991).

However, when fecundity of the species studied (as well as *H. castroi* and *H. pleoacuta*) is compared to the northern amphipods, it is observed that the mean number of eggs produced per female is far superior, as for *H. azteca* (Cooper, 1965; Hogg et al., 1995; Pilgrim and Burt, 1993; Strong, 1972) and *H. montezuma* (Oberlin and Blinn, 1997), being in accordance with the pattern of Sainte-Marie (1991). Therefore, our results suggest that in the southern freshwater amphipods latitude and climate do not influence the fecundity, and that this characteristic is more related to the females' body size.

5. Conclusions

In general, life history aspects of the studied amphipods showed similar patterns among species that occur closer to the tropics and among species from higher latitudes, supporting the hypothesis of this paper. However, some characteristics analyzed of the species from higher latitudes diverged from the

Northern Hemisphere amphipods, especially regarding the reproduction season, population abundance, body size and fecundity, refuting part of the proposed hypothesis. Thus, apparently there are patterns related to the latitude where amphipods occur, however, the regional climate and the habitat also seem to influence the population aspects of species of *Hyalella.*

In a country with continental dimensions and an unknown freshwater amphipods diversity like Brazil, use species of *Hyalella* from the North Hemisphere as a model of ecotoxicological studies and in evaluations of environmental quality is a mistake, since their population structures are very different from the Brazilian ones. The results of this study can assist in the creation of conservation programs for aquatic environments in Brazil, since these animals are endemics and extremely sensitive to environmental changes. This paper also collaborates with future studies of laboratory cultivation and ecotoxicology of *Hyalella*, because it provides information about the population structure of species in a natural environment.

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CONSIDERAÇÕES FINAIS

Os resultados obtidos nessa tese são pioneiros e servirão de base para futuros estudos com *Hyalella*, tanto no contexto ecológico, na conservação de ambientes dulcícolas ou puramente de cunho taxonômico. As informações sobre a filogenia do gênero na América do Sul são preliminares e para que se possa ter um pleno conhecimento da biogeografia, distribuição geográfica e das relações evolutivas de *Hyalella*, espécies dos países andinos, do oeste da Cordilheira dos Andes, Patagônia e Ilhas Malvinas devem ser estudadas. Além disso, futuramente, esses resultados poderão ser integrados com dados moleculares das espécies da América Central e do Norte, que já estão sendo obtidos por outros pesquisadores parceiros, de forma a se ter um pleno conhecimento da dispersão, distribuição e filogenia de *Hyalella*.

A ausência de fósseis de Amphipoda e da utilização de um grupo externo fora do gênero *Hyalella* nas análises filogenéticas pode ter subestimado o real tempo de origem e divergência do grupo, além da inferência das relações entre as espécies. Isso se deu devido à ausência de sequências disponíveis de anfípodos no GenBank que correspondessem ao fragmento dos genes utilizados nesse trabalho. Além disso, os primers comumente utilizados em estudos de *DNA barcoding* não funcionaram para os organismos de estudo, de forma que foi necessária a confecção de novos primers para a realização desse trabalho.

Muitas das espécies novas encontradas nessa tese já estão em processo de descrição, o que irá aumentar consideravelmente a diversidade de *Hyalella* no Brasil e na América do Sul. É aconselhável que populações de *Hyalella* que sejam futuramente descobertas passem por análises moleculares, visto que as características morfológicas nem sempre refletem as verdadeiras relações entre as espécies, especialmente se fizerem parte de um complexo de espécies.

Com relação ao estudo dos aspectos populacionais e ecológicos de espécies do gênero, os resultados encontrados servirão de base para pesquisas de ecotoxicologia, cultivo em laboratório e conservação desses animais. Foi possível observar que a ecologia e biologia desses anfípodos apresenta padrões similares entre espécies dos trópicos e entre espécies de altas latitudes, contudo, o ambiente físico e o clima influenciam muito na dinâmica e estrutura das populações. Apesar dos padrões observados, cada espécie apresenta características únicas e intrínsecas, que devem ser estudadas e aplicadas em programas de avaliação de qualidade ambiental e conservação.

Os resultados da tese foram inéditos, porém preliminares. Diversas regiões e estados brasileiros carecem de coletas desses anfípodos, bem como na maioria dos países da América do Sul. Trabalhos ecológicos e toxicológicos, amplamente realizados na América do Norte, com utilização das espécies de *Hyalella* em programas de conservação são ausentes ou ainda muito restritos no Brasil. Enfrentamos épocas de escassez de água e de grandes impactos ambientais nos ambientes dulcícolas no país, e estudos que visem o conhecimento dos organismos aquáticos, de suas relações com o ambiente e que ajudem em sua conservação e de todo o ecossistema são urgentes.