INSTITUTO DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA ANIMAL

EMÍLIA WELTER WENDT

Associação parasito-hospedeiro: abordagens filogenéticas, biogeográficas e coevolutivas em grupos de água doce no sudeste da América do Sul

PORTO ALEGRE
2020

UFRGS

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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Tese apresentada ao Programa de Pós-Graduação em Biologia Animal, Instituto de Biociências da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do título de Doutora em Biologia Animal.

Área de Concentração: Biologia Comparada
Orientador: Prof. Dr. Tiago Pinto Carvalho
Co-orientador: Prof. Dr. Luiz Roberto Malabarba

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Aprovada em 29 de abril de 2020.

BANCA AVALIADORA:

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Prof. Dr. Augusto Ferrari (FURG)

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Prof. Dr. Rogerio Vianna (FURG)

_____

PhD Brian L. Sidlauskas (Oregon State University)

Ao fechar das cortinas, me curvo e agradeço aos maiores incentivadores desse e de outros espetáculos dessa incrível jornada.
Dedico a vocês, com carinho, pai (*in memoriam*) e mãe.

"The most impressive aspect of the living world is its diversity….

… Wherever we look in nature, we find uniqueness"

Ernst Mayr

AGRADECIMENTOS

Todos sabemos ou já ouvimos falar o quanto a pós-graduação é difícil (ainda mais para quem leva realmente à sério), com muitos prazos para cumprir e uma incansável corrida pela excelência em publicações. Isso, as vezes, cansa! Eu cansei exatamente na metade do doutorado. Poderia simplesmente não falar disso aqui, mas sim, tive um quadro depressivo exatamente no meio do doutorado. Pensei em parar, em trocar de projeto, enfim, o fato é que nada disso importava naquele momento, pois era eu que precisava parar um tempo, desacelerar e me cuidar. Foi o que fiz e felizmente consegui superar, com a ajuda de muitas pessoas... família, amigos e professores. E é a todas essas pessoas que agradeço nesse momento. Essa tese tem um gostinho especial, sabe? E no final valeu muito a pena.

Preciso, em primeiro lugar, agradecer à algo ou alguém que não sei bem como nomear. Alguns chamam de Deus, Allah, Buda, outros de força do bem ou pensamento positivo...enfim, não importa, o fato é que algo me guia e me acompanha sempre! Obrigada!

Feito sim, agora sim posso me dirigir aos demais:

Agradeço ao meu orientador, prof. Dr. Tiago Pinto Carvalho, por ter aceito me orientar sem nem mesmo me conhecer, lá em 2016. Agradeço por todos esses anos de paciência, ensinamentos, incentivos e amizade. Mas agradeço principalmente pela confiança que tivestes em mim e no meu trabalho. Isso fez muita diferença para que eu continuasse. Saibas que és um exemplo de professor pra mim. Muito obrigada!

Agradeço ao meu co-orientador, prof. Dr. Luiz Malabarba, por abrir as portas do laboratório de Ictiologia para uma guria que só entendia de parasito (coragem!), e por me ensinar a admirar o mundo dos peixes. Mas agradeço principalmente por me incentivar a continuar e persistir na minha pesquisa, quando eu mesma duvidava da minha capacidade. Jamais vou esquecer do dia em que cheguei no laboratório dizendo que iria largar o doutorado, e tu, com toda calma disseste: "Não, não vais, teu trabalho é lindo. Tu só precisa de um descanso. Tudo vai dar certo". Muito obrigada Mala! És uma pessoa que admiro muito.

Agradeço à Coordenação de Aperfeiçoamento Pessoal (CAPES) pelo suporte financeiro (2016-2020), o qual garantiu que eu pudesse me dedicar exclusivamente à minha pesquisa e sem o qual não seria possível realizar essa tese. Agradeço também à Universidade Federal do Rio Grande do Sul (UFRGS), pela estrutura física.

Agradeço ao Programa de Pós-graduação em Biologia Animal da UFRGS, à sua coordenação (gestão atual e anterior) e a todos os docentes que fizeram parte do meu crescimento científico e pessoal.

Agradeço, com muito carinho, ao Dr. Walter Boeger e à Dra. Mariana Braga pela atenção durante esses quatro anos, e pelas valiosas colaboração nessa tese. Pelas discussões teóricas, pelos puxões de orelha, pelas análises científicas, pela paciência e pelos ensinamentos sobre a incrível relação entre os parasitos e seus hospedeiros.

Agradeço ao Dr. Michael Landis por ter me recebido com tanto carinho na Washington University, pela confiança e pelos valiosos ensinamentos sobre biogeografia. Agradeço também por me apresentar o melhor dunets dos Estados Unidos.

Agradeço aos meus pais, Guido Wendt (*in memoriam*) e Tereza Wendt, por serem exemplos de superação, amor e confiança. Obrigada por sempre acreditarem em mim. Essa tese é nossa. Amo vocês.

Agradeço os meus irmãos, Gui e Lu, e ao meu sobrinho, Pedro, pela confiança, amor, brigas, criticas, sugestões, auxilio emocional (e financeiro, muitas vezes), pelas longas risadas, e por estarem sempre comigo, mesmo de longe. Obrigada por serem quem são. Amo vocês.

Agradeço ao meu amor, Jeferson de Lima, por me apoiar em todos os momentos, por aceitar minhas ausências, e por me trazer para a realidade sempre que eu mergulhava demais nas minhas loucuras. Tua existência alegra minha vida de uma forma inigualável. Te amo!

Agradeço à minha segunda família, Sandra, Julio e Gui, pelo amor, carinho e incentivo de sempre.

Agradeço à minha terceira família, os amigos queridos, que felizmente são vário: Rê, Carol, Nanda, Saul, Ana (e Maluzinha), Débo, Filipe Abreu, Kelen (e Pedrinho), Filipe Michels, Sil, Laura Donin, Madoka, Barbara, Laurinha, Juju, Jucelio, Drose e demais amigos de Pelotas. E claro, aos queridos amigos da turma BBBritz2018.

Agradeço aos professores do laboratório de Ictiologia, Dr. Andre e Dra. Clarisse por todo suporte, ensinamentos e apoio durante todos esses anos.

Agradeço a todos os amigos do laboratório de Ictiologia (que também são vários!): Carol, Nanda, Juh, Juliano, Laura, Madoka, Victor, Dário, Junior, Julia, Mayara, Lorena, Rafa e a todos os alunos de iniciação científica. Obrigada pelas dicas no trabalho, ajuda no laboratório de molecular, ajuda com diafanizações (e diversas outras coisas sobre peixes), pelas coletas, pelas risadas e conversas jogadas fora na bancada.

Agradeço aos técnicos do departamento de Zoologia e queridos amigos, Tiago, Brenda e Valerí, os quais sempre estiveram prontos a ajudar da melhor forma possível.

Por fim, agradeço a todos que de alguma forma tornaram essa trajetória mais leve.

Muito Obrigada!

"Sou feita de retalhos"

Pedacinhos coloridos de cada vida que passa pela minha e que vou costurando na alma. Nem sempre bonitos, nem sempre felizes, mas me acrescentam e me fazem ser quem eu sou. Em cada encontro, em cada contato, vou ficando maior... Em cada retalho, uma vida, uma lição, um carinho, uma saudade... Que me tornam mais pessoa, mais humana, mais completa. E penso que é assim mesmo que a vida se faz: de pedaços de outras gentes que vão se tornando parte da gente também. E a melhor parte é que nunca estaremos prontos, finalizados... Haverá sempre um retalho novo para adicionar à alma. Portanto, obrigada a cada um de vocês, que fazem parte da minha vida e que me permitem engrandecer minha história com os retalhos deixados em mim. Que eu também possa deixar pedacinhos de mim pelos caminhos e que eles possam ser parte das suas histórias. E que assim, de retalho em retalho, possamos nos tornar, um dia, um imenso bordado de "nós". Cora Carolina

Sumário

Associação parasito-hospedeiro: abordagens filogenéticas, biogeográficas e coevolutivas em grupos de água doce no sudeste da América do Sul.

Emília Welter Wendt

Resumo: A presente tese utilizou abordagens multidisciplinares com o objetivo de reconstruir hipóteses filogenéticas para *Oligosarcus* e seus parasitos de brânquia, *Characithecium*, e estimar a provável história coevolutiva entre esses organismos. Para tal, primeiramente, o Capítulo I focou nas relações filogenéticas entre espécies de *Oligosarcus*, realizando uma estimativa de tempo de divergência para o gênero, bem como uma reconstrução ancestral de área. *Oligosarcus* é um grupo de peixes da família Characidae composto por 22 espécies, as quais possuem, principalmente, distribuíção alopátricas na região sudeste da América do Sul, e possuem ocorrência simpátrica para poucas espécies. Este gênero de peixe foi recuperado como monofilético, com alto suporte, e relacionado a linhagens atualmente atribuídas ao gênero *Astyanax*. Dentro de *Oligosarcus*, dois grupos com riqueza de espécies aproximadamente iguais são restritos principalmente às drenagens continentais e costeiras do sudeste da América do Sul. A radiação do gênero foi estimada para o Plioceno, com a maioria dos eventos de especiação ocorrendo durante o Pleistoceno. Estimativas da área ancestral utilizando métodos analíticos e modelos de evolução da paisagem (por exemplo, DIVALIKE e DEC) indicam a importância de barreiras fluviais (ex, as cataratas do Iguaçu) na bacia hidrográfica do rio da Prata e os efeitos das mudanças no nível do mar durante o Pleistoceno como moduladores de distribuições das espécies de *Oligosarcus*. Posteriormente, foi realizado no Capítulo II uma extensa investigação sobre a diversidade parasitária em brânquias de 17 espécies de *Oligosarcus*, bem como de 15 espécies de *Astyanax*, as quais são filogeneticamente próximas à *Oligosarcus* e com ocorrência simpátrica em muitos casos. Foram identificadas 7 espécies de *Characithecium*, sendo estas específicas de brânquias de *Oligosarcus* e *Astyanax*, e sendo recuperadas como monofiléticas a partir de dados moleculares. Além disso, foram investigadas se algumas características ecológicas estariam associadas a diferentes taxas de prevalências observadas para cada espécie de parasito em seus respectivos hospedeiros, e como diferentes caracteres morfológicos teriam evoluído dentro do gênero *Characithecium*. Após possuir esse conhecimento sobre as relações filogenéticas dos peixes (hospedeiros) e dos parasitos, e de identificar as associações entre esses indivíduos, essa tese finaliza com um estudo detalhado sobre a estrutura das interações entre parasitos e hospedeiros e a história coevolutiva dessas associações, bem como realiza uma estimativa de área ancestral para ambos os táxons. A partir de um estudo multidisciplinar, recuperamos a importância da oportunidade de contato entre os hospedeiros como mecanismo modulador da interação parasito-hospedeiro e o quanto isso demonstrou afetar a estruturação dessas redes. Em links com mais oportunidades de dispersão (= em bacias costeiras), a estrutura da rede era menos especializada do que nos links com poucas oportunidades de dispersão (= em bacias continentais). Além disso, devido a essa oportunidade, análises de ajuste global recuperaram várias expansões no número de hospedeiros utilizados como principais eventos coevolutivos que explicam a associação do *Characithecium* com seus

hospedeiros. Por fim, análises de reconstrução ancestral de área recuperaram dois cenários evolutivos para os parasitos. Em um deles utilizamos a informação de área ancestral dos hospedeiros (*Oligosarcus* e *Astyanax*) para restringir a área ancestral dos parasitos. Esse cenário recuperou a região costeira sul como área ancestral para *Characithecium*, e diversas dispersões posteriores a partir de 10 Ma. Por outro lado, um outro cenário, o qual foi realizado sem informações a priori sobre a distribuição dos hospedeiros, recuperou uma ampla área ancestral para *Characithecium*, indicando que esses parasitos provavelmente eram associados a outras espécies de peixes no início de sua radiação, as quais possuíam ampla dispersão. Nesse cenário, a associação com *Oligosarcus* e *Astyanax* teria ocorrido posteriormente a partir de novas colonizações e consequente extinção nos hospedeiros ancestrais.

Palavras-chave: interação parasito-hospedeiro, expansão no número de hospedeiros, nova colonização, coevolução, peixes e parasitas.

Host-parasite association: phylogenetic, biogeographic and coevolutionary approaches in freshwater groups in southeastern South America.

Emília Welter Wendt

Abstract: This thesis used multidisciplinary approaches in order to reconstruct phylogenetic hypotheses for *Oligosarcus* and *Astyanax*, and their gill parasites, *Characithecium*, and to estimate the probable coevolutionary history between these organisms. First, Chapter I focused on the phylogenetic relationships between species of *Oligosarcus*, making an estimate of the divergence time for the genus, as well as an ancestral area reconstruction. *Oligosarcus* is a group of Characidae composed of 22 species, which have mainly allopatric distribution in the southeastern region of South America and have a sympatric occurrence for a few species. This fish genus was recovered as monophyletic, with high node support, and related to lineage currently attributed to the *Astyanax* genus. Within *Oligosarcus*, two groups with approximately equal species richness were resolved as monophyletic, restricted mainly to continental and coastal drainages in southeastern South America. The radiation of the genus was estimated for the Pliocene, with most speciation events occurring during the Pleistocene. Estimates of the ancestral area using analytical methods (e.g., DIVALIKE and DEC) indicate the importance of river barriers (e.g., Iguaçu waterfalls) in the La Prata basin and the effects of sea-level changes during the Pleistocene for the distributions of the *Oligosarcus* lineage. Subsequently, an extensive investigation was carried out in Chapter II on the parasitic diversity in gills of 17 species of *Oligosarcus*, as well as 15 species of *Astyanax*, which are phylogenetically close to *Oligosarcus* and with sympatric occurrence to some species of that genus. Seven species of *Characithecium* were identified, these being specific to gills of *Oligosarcus* and *Astyanax*, and being recovered as monophyletic from molecular data. In addition, it was investigated whether some ecological characteristics would be associated with different prevalence rates observed for each parasite species in their respective hosts, and how different morphological characters would have evolved within *Characithecium*. After having knowledge about the phylogenetic relationships of fish (hosts) and parasites, and identifying the associations between these individuals, this thesis presents a detailed study on the structure of interactions between parasites and hosts and the coevolutionary history of these associations, as well how to estimate ancestral area for both taxa. From a multidisciplinary study, we recovered the importance of the opportunity for contact between hosts as a modulator mechanism of the host-parasite interaction and how much this has been shown to affect the structuring of these networks. In links with more opportunities for dispersion (= coastal links), the network structure was less specialized than in links with few opportunities for dispersion (= continental links). In addition, due to this opportunity, global-fit analyses recovered several host-range expansions as main coevolutionary events that explain the association of *Characithecium* with its hosts. Finally, analyzes of ancestral area reconstruction recovered two evolutionary scenarios for the parasites. In one of them, we used the ancestral area information of the hosts (*Oligosarcus* and *Astyanax*) to restrict the ancestral area of the parasites. This scenario recovered the

southern coastal region as an ancestral area for *Characithecium*, and several dispersals after 10 Ma. On the other hand, another scenario, which was carried out without prior information on the distribution of the hosts, recovered a wide ancestral area for *Characithecium*, indicating that these parasites were probably associated with other fish species at the beginning of their radiation, which had wide dispersion. In this scenario, the association with *Oligosarcus* and *Astyanax* would have occurred later on from new colonizations and consequent extinction in the ancestral hosts.

Keywords: host-parasite interaction, host-range expantion, new colonization, coevolution, fish and parasite.

CAPÍTULO INTRODUTÓRIO

Introdução Geral

Oligosarcus: relações filogenéticas

Characidae é a família mais diversa e com a maior complexidade filogenéticas dentro de Characiformes, correspondendo 58% de toda a diversidade dentro da ordem (Weitzman & Malabarba,1998; Mirande, 2010; Oliveira *et al.*, 2011). Dentro dessa diversa família de peixes, *Oligosarcus* Günther, 1864 contribui com 22 espécies, as quais se distribuem amplamente na América do Sul, abrangendo os territórios do Brasil, Uruguai, Argentina, Bolívia, Peru e Paraguai (Menezes, 1988; Ribeiro & Menezes, 2015; Menezes & Ribeiro, 2015).

Hipóteses de relacionamento deste gênero com outros Characiformes, primeiramente, propuseram *Acestrorhynchus* Eigenmann & Kennedy, 1903 como grupo irmão e ambos inseridos na tribo Acestrorhynchini (Menezes, 1969). Mais tarde, Buckup (1998) invalidou esta proposta e sugeriu que ambos os gêneros supracitados eram distantes filogeneticamente. Posteriormente, *Astyanax* e *Bramocharax* foram propostos como filogeneticamente próximos a *Oligosarcus*, baseando-se em dados morfológicos (Mirande, 2010; Mirande *et al*., 2011) e moleculares (Oliveira *et al*., 2011). Recentemente, com auxílio de uma análise de evidencia total, Mirande (2018) propôs novos relacionamentos dentro da subfamília Stethaprioninae (Characidae), sendo esta composta por quatro grades tribos. Nesse trabalho, *Oligosarcus* foi recuperado dentro de um grande clado juntamente com espécies de *Astyanax*, *Hyphessobrycon* Durbin, 1908, *Hasemania* Ellis, 1911, e *Gymnocharacinus* Steindachner, 1903, e sendo este grande clado o grupo irmão de *Astyanax sensu* Mirande (2018).

As hipóteses de relacionamento dentro de *Oligosarcus*, até recentemente, eram baseadas principalmente em dados morfológicos (Mirande, 2010; Mirande et al., 2011; Almirón et al., 2015; Ribeiro & Menezes, 2015), e discordam quanto a algumas sinapomorfias para o gênero. *Oligosarcus sensu* Mirande et al. (2011), possui a presença de duas fileiras de dentes na pré-maxila, enquanto *Oligosarcus sensu* Ribeiro & Menezes (2015) possui apenas uma fileira de dentes. A proposta de Mirande et al. (2011) permitiu a inclusão de três espécies dentro de *Oligosarcus*, tais como: *Oligosarcus itau* Mirande et al., 2011, *Oligosarcus amome* Almirón, Casciotta, Piálek, Doubnerová e Rican 2015 e *Oligosarcus platensis* (Messner, 1962). No entanto, a proposta mais abrangente para o gênero, discorda quanto ao posicionamento dessas três espécies dentro de *Oligosarcus* (Ribeiro & Menezes, 2015). Esse estudo incluiu 18 espécies de *Oligosarcus* e baseou-se

em 34 caracteres morfológicos testados em uma estrutura de parcimônia, a qual recuperou a monofilia para o gênero (Ribeiro & Menezes, 2015).

Nesse sentido, para elucidar tais relações filogenéticas, análises moleculares tem contribuído intensamente com pesquisas na área taxonômica (Oliveira *et al*. 2011). Os resultados podem confirmar parentescos já estabelecidos pelas análises morfológicas ou podem trazer novas hipóteses de relacionamento (Meyer & Zardoya, 2003). Para *Oligosarcus*, estudos incluindo informações moleculares incluíaram poucas espécies, e tinham o objetivo principal de posicionar o gênero dentro de Characidae (Ortí & Meyer, 1997; Javonillo et al., 2010; Oliveira et al., 2011; Betancur et al., 2018) ou delimitar espécies em estudos regionais utilizando o código de barras (Pereira et al., 2011; Carvalho et al., 2011; Rosso et al., 2012; Pereira et al., 2013; Diaz et al., 2016). Logo, tais estudos contribuíram pouco para o entendimento sobre as relações de parentesco entre as espécies de *Oligosarcus*, bem como para a história evolutiva do gênero como um todo.

Nesse sentido, o **Capítulo I** dessa tese utilizou ferramentas moleculares para acessar o posicionamento das espécies dentro do gênero. Foi incluída 77% da diversidade de *Oligosarcus*, amostrados em uma ampla distribuição geográfica. Além disso, estimativas de tempo de divergência juntamente com análises biogeográficas, permitiu a investigação e a apresentação de uma hipótese evolutiva para *Oligosarcus* na região sudeste da América do Sul.

Relação parasito-hospedeiro e a filogenia de *Characithecium*

O parasitismo é o modo de vida adotado por uma parcela significativa dos organismos, ocorrendo em diversos ecossistemas e nos mais variados grupos de hospedeiros (Combes, 1995). Sabe-se que a diversidade parasitária em peixes de água doce é bastante considerável, sendo distribuída em seis grandes táxons, Trematoda, Monogenoidea, Cestoda, Acanthocephala, Nematoda e Crustacea, com registro de aproximadamente 1.050 espécies de parasitos presentes em cerca de 620 espécies de peixes de água doce neotropical (Eiras et al., 2010).

No entanto, a fauna parasitária, especialmente com relação aos Monogenoidea, é praticamente desconhecidos para o gênero *Oligosarcus*. Um estudo anterior relatou cinco espécies de parasitos do gênero *Characithecium* ocorrendo em brânquias de *Oligosarcus jenynsii* (Günther, 1864), sendo quatro delas descritas e até o momento encontradas apenas neste hospedeiro (Rossin & Timi, 2015).

Este gênero faz parte da família Dactylogyridae Bychowsky, 1933 e é formado atualmente por sete espécies as quais parasitam brânquias de peixes de água doce, distribuídos em bacias hidrográficas da América Central (México e Panamá) e América do Sul (Colômbia, Brasil, e Argentina). Além do registro de espécies de *Characithecium* em *O. jenynsii*, esse gênero foi registrado em poucas espécies de *Astyanax* [*Astyanax aeneus* (Gunther, 1860), *Astyanax ruberrimus* Eigenmann, 1913, *Astyanax fasciatus* (Cuvier, 1819), *Astyanax lacustris* (Lutken, 1875) e *Astyanax scabripinnis* (Jenyns, 1842)] (Kritsky & Leiby, 1972; Gioia et al., 1988; Boeger & Vianna, 2006; Gallas et al., 2016).

Apesar de ser composto por poucas espécies, nenhuma hipótese filogenética foi proposta para *Characithecium*, e também permanece desconhecida a ocorrência desses parasitos em congêneres de *O. jenynsii*. Da mesma forma, é desconhecida a história evolutiva de *Characithecium*, e como estes parasitos interagem com seus hospedeiros. Dados anteriores sugeriram uma provável relação coevolutiva entre esses parasitos e peixes dos gêneros *Oligosarcus* e *Astyanax* (Rossin & Timi, 2015), devido principalmente, à proximidade filogenética desses peixes (Mirande, 2010; Mirande *et al*., 2011; Oliveira *et al*., 2011). No entanto, essa hipótese ainda não foi avaliada cientificamente.

Nesse sentido, no **Capítulo II** encontramos um estudo detalhado sobre a amplitude de ocorrência das espécies de *Characithecium* em diversas espécies de *Oligosarcus* e *Astyanax*, abrangendo uma ampla área geográfica. Ainda, esse capítulo fornece informações filogenéticas sobre o gênero *Characithecium*, baseando-se em dados moleculares, e apresenta um estudo de delimitação de espécies. Além disso, foram estimadas quais variáveis ecológicas estão associadas às diferentes taxas de prevalência encontradas nos diversos hospedeiros e como os principais caracteres morfológicos evoluíram dentro do gênero.

História biogeográfica do sudeste da América do Sul: utilizando *Oligosarcus* e seus parasitos como modelo de estudo.

Sabe-se que a evolução dos peixes de água doce está fortemente ligada à história geológica das drenagens que habitam, devido ao isolamento desses organismos em bacias hidrográficas. Nesse sentido, a plataforma Sul Americana passou por diversas mudanças desde o período de separação da Gandwana até o presente, onde a elevação do escudo cristalino e a formação das bacias costeiras foram consequências diretas de

movimentações tectônicas, as quais moldam o senário atual de distribuição dos peixes nessa região (Ribeiro, 2006). Estima-se que durante o Terciário houvessem pontos de intercâmbio entre a fauna do escudo cristalino e da região costeira, fato este que possibilitou a dispersão dos peixes (Ribeiro, 2006).

A região costeira da América do Sul é conhecida pelo alto grau de endemismo para peixes de água doce (Weitzman et al., 1988), bem como pela presença de uma complexa história evolutiva e de formação geológica. Essa região é formada por inúmeras bacias hidrográficas ao longo da costa brasileira, as quais são atualmente isoladas umas das outras (Thomaz & Knowles, 2018) e isoladas das drenagens continentais a partir de uma formação montanhosa escarpada ao longo da face leste do escudo cristalino brasileiro (Ribeiro, 2006). A configuração biológica da região costeira teria sido moldada durante o Pleistoceno, onde, eventos de transgressão e regressão do nível do mar alteraram as conexões entre as drenagens. Durante períodos interglaciais, com o aumento no nível do mar, parte das drenagens anteriormente conectadas ficaram submersas, sendo conhecidas como paleodrenagens (Thomaz & Knowles, 2018). Esse evento aconteceu pelo menos quatro vezes e promoveu isolamentos e conexões de drenagens, influenciando na evolução de inúmeras espécies de peixes (Weitzman et al., 1988; Thomaz et al., 2015, 2017; Thomaz & Knowles, 2018).

Por outro lado, as drenagens continentais formam grandes regiões de inundação, como por exemplo o Alto Paraná, Paraguai, Chaco, Baixo Paraná e Uruguai. Essas drenagens continentais são isoladas exclusivamente por quedas d'água, as quais formam barreiras de dispersão para os peixes. Um exemplo disso são as cataratas do Iguaçu, que se formaram à aproximadamente 2 milhões de anos atrás e isolam a bacia do Iguaçu das demais drenagens da bacia do La Plata (Stevaux & Latrubesse, 2010), contribuindo para inúmeras espécies endêmicas na bacia do Iguaçu (Abell et al., 2008; Baumgartner et al., 2012).

Oligosarcus se distribui amplamente pela região sudeste da América do Sul, ocorrendo tanto em bacias continentais como ao longo da região costeira. Uma grande parte das espécies se distribui de forma alopátricas, e algumas poucas se distribuem de forma simpátrica (Menezes, 1988; Ribeiro & Menezes, 2015; Menezes & Ribeiro, 2015). Além disso, as espécies parecem possuir diferentes preferências ecológicas, as quais influenciam a distribuição geográfica do gênero. Menezes (1988), separou as espécies em duas categorias, "upland" e "lowland", baseado na ocorrência das espécies em rios de cabeceira e rios de planície, o que parece isolar algumas espécies devido a essa

preferência de hábitat. Somado a isso, a distribuição das espécies de *Oligosarcus* parece se diferenciar entre ambientes lacustres e ribeirinhos. Baseado nessas distribuições das espécies de *Oligosarcus*, dois principais processos biogeográficos são apontados como moduladores da distribuição e evolução do gênero, tais como: a vicariância (explicada pelo elevado número de espécies alopátricas) e a reticulação (permitindo a dispersão entre drenagens anteriormente isoladas e criando oportunidades de simpatria entre espécies distantes dentro do gênero *Oligosarcus*).

Somando nisso, a presente tese buscou estimar processos biogeográficos e entender como as espécies de *Oligosarcus* teriam respondido a eles. Da mesma forma, espécies de parasitos intimamente ligados a esses peixes podem ter enfrentado as mesmas barreiras geográficas e terem sua evolução influenciada por isso. Para tal, espécies que possuem alta especificidade a seus hospedeiros, como os monogenoideos, são frequentemente escolhidas como modelos de estudo. Monogenoidea (*sensu* Bychowsky, 1937) são parasitos obrigatórios, ocorrendo principalmente em peixes de água doce (Boeger & Vianna, 2006; Cohen *et al*., 2013), e devido a sua alta especificidade parasitária, são frequentemente utilizados para investigar sua interação com os hospedeiros, bem com sua relação biogeográfica (Domingues & Boeger, 2005; Mendlová & Šimková, 2014; Braga et al., 2015; da Graça et al., 2018).

Sabe-se que a história evolutiva dos hospedeiros pode influenciar na evolução dos parasitos (Filipiak *et al*., 2016). Essa evolução conjunta entre dois ou mais táxons é conhecida como coevolução e vem sendo intensamente estudada nos últimos anos (Boeger & Kritsky, 1997; Ronquist, 1997; Balbuena *et al*., 2013; Martínez-Aquino *et al*., 2014; Hahn *et al*., 2015; Fountain *et al*., 2017). Diversos processos coevolutivos estão associados a essa evolução conjunta, tais como coespeciação, duplicação (especiação dentro do hospedeiro), inércia (falha na especiação do parasito quando da especiação do hospedeiro), troca de hospedeiros e extinção (Brooks, 1987; Boeger & Kritsky, 1997).

Devido a todas as características mencionadas, Monogenoidea e peixes de água doce tornam-se ótimos sistemas biológicos para estudar processos coevolutivos e biogeográficos. Para tal, no **Capítulo III** foram utilizados esses organismos com o objetivo de estimar a área ancestral dos peixes e dos parasitos e entender como esses organismos evoluíram na complexa região do sudeste da América do Sul.

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CAPÍTULO I

Published in Molecular Phylogenetics and Evolution

ISSN: 1055-7903

Phylogenetic relationships and historical biogeography of *Oligosarcus* (Teleostei: Characidae): examining riverine landscape evolution in southeastern South America

Emília W. Wendt, Priscilla C. Silva, Luiz R. Malabarba, Tiago P. Carvalho

Volume 140, 106604

DOI: https://doi.org/10.1016/j.ympev.2019.106604

Molecular Phylogenetics and Evolution 140 (2019) 106604

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Phylogenetic relationships and historical biogeography of *Oligosarcus* (Teleostei: Characidae): Examining riverine landscape evolution in southeastern South America

Emília W. Wendt[a,*], Priscilla C. Silva[a,b], Luiz R. Malabarba[a], Tiago P. Carvalho[a,c]

[a] Laboratório de Ictiologia, Departamento de Zoologia, Universidade Federal do Rio Grande do Sul – UFRGS, Av. Bento Gonçalves, 9500, Bloco 4, prédio 43.435, 91501-970 Porto Alegre, RS, Brazil

[b] Laboratório de Sistemática Molecular-Beagle, Departamento de Biologia Animal, Universidade Federal de Viçosa – UFV, Av. Ph. Rolfs, sn, Anexo II CCB, 36570-000 Viçosa, MG, Brazil

[c] Laboratorio de Ictiología, Unidad de Ecología y Sistemática (UNESIS), Departamento de Biología, Facultad de Ciencias, Pontificia Universidad Javeriana, Carrera 7 N° 43-82, Bogotá D.C., Colombia

ARTICLEINFO

Keywords:
Freshwater fishes
Multilocus analysis
Paleogeography
Species Tree analysis
Ancestral range estimation
Landscape evolution models

ABSTRACT

The pike-characin *Oligosarcus* is a group of Characidae composed of 22 species, which have mostly allopatric distributed species in southeastern South America and sympatric occurrence of few species. *Oligosarcus* shares a similar distribution pattern with other fish genera and therefore, can help us to understand biogeographic events that influenced freshwater fish distribution in the southeastern South America. Our paper presents the most extensive taxonomic coverage for molecular analysis of *Oligosarcus* and uses various methods to examine the evolutionary history of the genus. Phylogenetic relationships among species of *Oligosarcus* were examined using a multilocus dataset by Maximum Likelihood and Bayesian methods. A relaxed molecular clock was used to estimate lineage divergence times, which provide a framework to examine the biogeographic history of this clade across the drainage basins of southeastern South America. *Oligosarcus* was resolved as monophyletic with strong support, and related to lineages currently assigned to the genus *Astyanax*. Within *Oligosarcus*, two groups of approximately equal species richness were resolved as monophyletic, mainly restricted to continental and coastal drainages of southeastern South America. *Oligosarcus* radiation is estimated to the late Neogene, with its origin in the Pliocene and most speciation events occurring in the Pleistocene. Some apomorphic characteristics associated with piscivory (e.g. large caniniform teeth) in Oligosarcus likely have evolved once, and are convergent to similar phenotypes observed in a distantly related clade of *Astyanax* (formerly *Bramocharax*). In addition, the presence of morphological convergence within the genus *Oligosarcus* (e.g. trophic morphology) seems to explain the difference between the present molecular hypothesis and some previous morphological studies. Ancestral geographical range estimation using analytical methods (e.g. DIVALIKE and DEC) demonstrated the effects of different Landscape Evolution Models (LEMs) on diversification of Oligosarcus. The results suggest that the two main Oligosarcus clades evolved in allopatry in continental and coastal drainages, with subsequent range extension and vicariance events that established the modern distributions. LEM analyses indicate the importance of formation of riverine barriers across the watershed of the La Plata basin and the effects of sea-level changes during the Pleistocene for delineating lineage distributions of *Oligosarcus*.

Introduction

Biogeographic studies seek to understand patterns in the distribution of species and how they were generated, based on geological history and evolutionary events (Posadas et al., 2006). Therefore, the evaluation of biogeographical processes and phylogenetic relationships collaborate to a better understanding of the biogeographical history of taxa. More recently, the search for more realistic models and their implementation on biogeography have supported more sophisticated and robust studies, making use of geological data on hypothesis testing frameworks (Ree et al., 2005; Landis et al., 2013; Matzke, 2013; Ree and Sanmartin, 2018). These models take into account biogeographic events such as dispersion, vicariance, and within-area speciation, which are estimated based on the relationships between species and their current distributions (Matzke, 2013). Finally, the best-fit model is selected, which can better explain the evolution of the taxon studied in a model-fitting framework. In this sense, freshwater fish are great models for biogeographic studies due to their limited dispersal capacity between drainage basins, which change from geomorphological reconfiguration (Lundberg et al., 1998; Ribeiro, 2006; Albert et al., 2011; Dagosta and de Pinna, 2017).

The biogeographic history of southeastern South America indicates that freshwater fish distribution is constrained mostly by two main events: (1) drainage reconfiguration during Neotectonic fault activation in the Quaternary (*e.g.* river captures between continental and coastal drainages and barrier formation, causing, respectively, connection and isolation of lineage, Ribeiro, 2006; Ribeiro et al., 2016; Stevaux and Latrubesse, 2010), and (2) sea-level fluctuations that promoted paleodrainage connections and isolations during the Pleistocene (Weitzman et al., 1988; Thomaz et al., 2015b, 2017; Thomaz and Knowles, 2018). In other words, headwater capture, barrier formation and drainage connection/isolation associated with sea-level changes can promote freshwater faunal exchanges, fostering lineage dispersal and speciation (Aquino and Colli, 2016; Thomaz et al., 2017). Biogeographic studies using fish as models seek to understand the dynamics of these events and associate them with congruent patterns of species distribution (Ribeiro, 2006; Lima and Ribeiro, 2011; Tagliacollo et al., 2015; Thomaz et al., 2015b, 2017; Lima et al., 2017; Machado et al., 2018). More recently historical biogeography on freshwater fishes has been trying to examine the influence of geological process as landscape evolution models constraining range evolution and contrast them into a different hypothesis of past riverine connections using model-based

approaches (Bossu et al., 2011; Tagliacollo et al., 2015; Machado et al., 2018). Based on it's broad and mostly allopatrically distributed species in southeastern South America, and the sympatric occurrence of few species, *Oligosarcus* Günther, 1864 is an exciting group to investigate biogeographical processes delineating species distribution in this region (Menezes, 1987; 1988; Ribeiro and Menezes, 2015). In addition, *Oligosarcus* shares a similar distribution pattern with other fish genera in the region (*e.g. Mimagoniates*, *Phalloceros*, *Diapoma* and *Bryconamericus*; Camelier et al., 2018; Thomaz et al., 2015; 2019) and therefore can shed light to general biogeographical patterns.

Oligosarcus is a group of Characidae fish composed of 22 species with small to medium body sizes (Menezes, 1987; Miquelarena and Protogino, 1996; Mirande et al., 2011; Almirón et al., 2015; Ribeiro and Menezes, 2015), and with *Oligosarcus argenteus* Günther, 1864 as its type species. *Oligosarcus* is mostly piscivore (Hermes-Silva et al., 2004; Abelha et al., 2012), but some species have diets based on aquatic and terrestrial insects and other arthropods (Menezes, 1969; Casatti, 2003; Hermes-Silva et al., 2004; Araujo et al., 2005). This genus is distributed throughout most of southeastern South American river basins, including two species endemic to the Bolivian and Argentinean Andean piedmont. The remaining species occur in the Brazilian crystalline shield, lowland areas of the La Plata Basin, and coastal rivers of south and eastern Brazil (Ribeiro and Menezes, 2015). More particularly, most of the allopatric species are commonly found in upland areas, whereas lowland species tend to be sympatric with other congeners (Menezes, 1988; Ribeiro and Menezes, 2015). Previous hypotheses on the biogeography of *Oligosarcus* suggested that the primary process delimitating species distribution was vicariance associated with barrier formation (Menezes, 1988) and that the genus started its radiation in upland regions and later dispersed to lowlands (Ribeiro and Menezes, 2015).

Previously proposed phylogenetic relationships of *Oligosarcus* within Characiformes are not congruent (Menezes, 1969; Buckup, 1998; Mirande, 2009; Mirande, 2018). *Oligosarcus* was reported close to *Acestrorhynchus* within Acestrorhynchini (Menezes, 1969), a hypothesis subsequently refused by Buckup (1998) based on morphology, suggesting *Oligosarcus* closely related to the clade composed by *Tetragonopterus* (*Phenacogaster* (*Charax + Cynopotamus*)). In the CLOFFSCA (Reis et al., 2003), *Oligosarcus* was placed as an "*incertae sedis*" genus in Characidae (Lima et al., 2003). Later, based on a character-rich cladistics analysis of morphological data,

Oligosarcus was hypothesized to be closely related to the Central American *Bramocharax* Gill, 1877 (Mirande, 2009; Mirande, 2010; Mirande et al., 2011). Regarding molecular data, *Oligosarcus* is closely related to *Astyanax* Baird & Girard, 1854 (Ortí and Meyer, 1997; Javonillo et al., 2011; Oliveira et al., 2011; Betancur et al., 2018). More recently, in a total-evidence analysis, Mirande (2018) proposed a new tribe (Gymnocharacini) within the subfamily Stethaprioninae, where *Oligosarcus* forms a group within a large clade that also includes species of *Astyanax*, *Hyphessobrycon* Durbin, 1908, *Hasemania* Ellis, 1911, and *Gymnocharacinus* Steindachner, 1903, and being this large clade a sister group of the *Astyanax* clade *sensu* Mirande (2018) including the type species of the genus.

The most species-comprehensive phylogenetic analyses of *Oligosarcus* are based on morphology only (Mirande, 2010; Mirande et al., 2011; Almirón et al., 2015; Ribeiro and Menezes, 2015). Ribeiro and Menezes (2015), using 34 morphological characters in a parsimony framework, recovered *Oligosarcus* as monophyletic group (Figure 1) supported by having premaxillary teeth in a single row and two larger fang-like caniniform teeth and by having tricuspid teeth in the ectopterygoid bone. Generic status controversies remain about the inclusion of some species within *Oligosarcus*, such as *Oligosarcus itau* Mirande, Aguilera & Azpelicueta, 2011, *Oligosarcus amome* Almirón, Casciotta, Piálek, Doubnerová & Rican 2015, and *Oligosarcus platensis* (Messner, 1962) that have synapomorphic features of *Oligosarcus* (*sensu* Mirande, 2011), but have two rows of teeth in the premaxilla (vs. one row in the remaining *Oligosarcus* species). In contrast, molecular phylogenetic analyses including *Oligosarcus* are species-poor and aimed to position the genus within Characidae (Ortí and Meyer, 1997; Javonillo et al., 2010; Oliveira et al., 2011; Betancur et al., 2018), or to delimit species in regional barcode studies (Pereira et al., 2011; Carvalho et al., 2011; Rosso et al., 2012; Pereira et al., 2013; Barros et al., 2015; Diaz et al., 2016).

The close relationship between *Oligosarcus* and *Astyanax* species calls for inquiry on putative convergence of characters, similar to the observed condition in the pike-like characiform genus *Bramocharax* (currently a junior synonym of *Astyanax*; Ornelas-Garcia et al., 2008; Schmitter-Soto, 2016, 2017 and Garita-Alvarado et al., 2018) and *Astyanax* species in Central America. These authors studied the phylogenetic relationships between species of *Astyanax* and "*Bramocharax"* and supported the polyphyly of the latter. Four species were traditionally included in "*Bramocharax*" because they shared morphological characteristics related to dentition and body shape,

that may be associated with adaptive convergences linked to ecological factors such as habitat and diet (Ornelas-Garcia et al., 2008; Schmitter-Soto, 2016; Garita-Alvarado et al., 2018).

In this paper we (1) investigate phylogenetic relationships and divergence time estimates in *Oligosarcus* using a species trees and a fossil calibrated molecular phylogeny, (2) examine the influence of landscape evolution on biogeographic processes that shaped species distribution in southeastern South America and (3) comment on the nature of convergent characters associated with piscivory. This paper presents the broadest taxonomic coverage for molecular analysis of the genus *Oligosarcus* and uses various methods to examine the evolutionary history of the genus. Therefore, new interspecific relationships and biogeographic analyses of *Oligosarcus* can help us to understand biogeographic events that influenced freshwater fish distribution in the southeastern region of South America.

FIGURE 1.

2. Material and methods

2.1 *Taxonomic sampling*

Molecular data of 152 specimens were used for phylogenetic reconstructions. Of those, 65 specimens represented 17 species of *Oligosarcus*, corresponding to 77% of the 22 valid species in the genus (supplementary material 1, Table S1). Tissue samples were obtained from the following museum collections: Universidade Estadual Paulista, São José do Rio Preto (DZSJRP); Universidade Estadual Paulista, Botucatu (LBP); Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre (MCP); Museu de Zoologia, Universidade Estadual de Londrina, Londrina (MZUEL); Universidade Federal do Rio Grande do Sul, Porto Alegre (UFRGS); and Coleção Zoológica da Universidade Federal do Mato Grosso do Sul, Campo Grande (ZUFMS). Sampling includes, when possible, specimens of *Oligosarcus* from different river basins representing species distribution broadly. Specimens of *Oligosarcus* were identified based on diagnostic morphological traits proposed by Menezes (1988), Mirande et al. (2011), Almirón et al. (2015), Menezes and Ribeiro (2015), and Ribeiro and Menezes (2015).

The outgroup was chosen based on previous studies, both molecular and morphological, that report close relationship between *Oligosarcus* and *Astyanax* (Ortí and Meyer, 1997; Mirande, 2010, 2018; Javonillo et al., 2011; Oliveira et al., 2011; Betancur

et al., 2018) and also several representatives of Characidae and related families. It included 37 specimens representing 24 species of *Astyanax*, mostly representatives of *Astyanax* clade *sensu* Mirande (2018), but also other species traditionally included in *Astyanax sensu* Eigenmann, 1917 (Lima et al., 2003), in addition to 38 species of Characidae and closely related families. Sequences of extant species, which were suggested as closely related to fossil taxa, were included in the analyses to perform a node-based time calibration on the phylogenetic tree (see Table S1 and subtopic 2.4). Sequences of most species of *Astyanax* for *ND2*, *COI*, and *MYH6* genes were obtained from Silva (2017) and Silva et al. (2019). Other outgroup sequences were obtained from previously published phylogenies (*e.g*. Javonillo et al., 2010; Oliveira et al., 2011; Hirschmann et al., 2015; Thomaz et al., 2015a; Mirande, 2018; Table S1) via GenBank. A complete list of tissues and specimen vouchers is given in Table S1.

2.2. *DNA extraction, amplification, sequencing and alignment*

Tissue samples were preserved in 99% ethanol at either -80°C or -18°C. DNA extraction from tissues followed a modified CTAB protocol (Doyle & Doyle, 1987). Polymerase chain reaction (PCR) was used to amplify two mitochondrial and three nuclear markers. Partial sequences of mitochondrial markers cytochrome c oxidase subunit 1 - *COI* [~ 714 bp] and NADH dehydrogenase 2 - *ND2* [~ 1000 bp]), and nuclear markers (Recombination activation gene 2 – *RAG2* [~1083 bp], alpha-myosin 6 - *MYH6* [~782 bp] and intron I of the *S7* ribosomal protein gene [~743 bp]) were included in the analyses.

Gene *COI* was amplified using the primers proposed by Ivanova et al. (2007) and Melo et al. (2011). To amplify the *ND2* sequences, a set of primers (ND2-F and ND2-R) was developed (Table S2) based on a complete sequence of the gene *ND2* of *Oligosarcus argenteus*. For this, the software Primer3Plus (Untergasser et al., 2007) was used, and the quality of the primer was tested in the software NetPrimer (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html). To amplify the *RAG2* sequences, the set of primers of Oliveira et al. (2011) were used in a cocktail, performing a single PCR. The *MYH6* and S7 genes were amplified using the primers proposed by Li et al. (2007) and Chow and Hazama (1998), respectively. A list with of all the primers used in this study is presented in Table S2. DNA fragments were amplified by PCR in 20 μL reactions accordingly: 10-50 ng DNA, 0.2 μL of each primer at 10μM of, 0.2 mM of each dNTP, 1X Buffer, 1.5 μM MgCl2 and 1U Platinum Taq DNA polymerase (Invitrogen, São Paulo, SP, Brazil). PCR products were checked by

electrophoresis in agarose gel, purified using ExoSap (Exonuclease I and Shrimp Alkaline Phosphatase GE Healthcare®, Piscataway, NJ, USA) and sequenced in both directions by Macrogen Inc (Seoul, South Korea) and ACTGene (Porto Alegre, Brazil). Forward and reverse sequences were visually inspected, edited, and combined into contigs using the software Geneious 8.0 (Kearse et al., 2012). PCR conditions for all markers are found in Table S2.

Sequences were aligned using the MUSCLE algorithm (Edgar, 2004) embedded in the software Geneious 8.0 under default parameters. Alignments of coding regions were visually inspected to verify that all sequences follow the correct reading frame and do not contain stop codons. The five markers were concatenated into a single matrix for phylogenetic analyses (except for Species Tree analyses; see below). Whenever uncertainty of nucleotide identity was detected in the chromatograms, IUPAC ambiguity codes or "N" were applied. Sequences were deposited in GenBank (Table S1).

2.3. *Phylogenetic reconstruction*

Nucleotide substitution models and partition schemes were evaluated using PartitionFinder v1.1.1 (Lanfear et al., 2012) (Table S3). Genes were partitioned by codon position (except for the intron S7), and best partition scheme was selected using the Bayesian Information Criterion, evaluating specific substitution models for each of the software used in phylogenetic reconstructions. Phylogenetic relationships were performed using Maximum Likelihood (concatenated matrix) and Bayesian Inference (to individual genes, concatenated matrix, and Species Tree analyses).

The Maximum Likelihood (ML) analyzes ran in RAxML v2.0.1 (Stamatakis, 2006), and the evolutionary model used for data blocks was GTRGAMMA. RAxML searches were conducted in the CIPRES portal (Miller et al., 2010) using ten parallel runs and starting with a randomly generated tree. Branch support was assessed using the thorough bootstrap algorithm with 1000 replicates.

Phylogenetic relationships of individual gene trees and concatenated dataset were estimated in MrBayes 3.2.2. (Ronquist et al., 2012). Individual gene tree analyses were performed to examine the influence of each marker on the topology. Two runs of four chains were conducted simultaneously over 40,000,000 generations with sample frequency every 4,000 generations and 10,000,000 generations with sample frequency every 1,000 generations for the concatenated tree and gene tree analyses, respectively. *Serrasalmus* sp. was used to root the tree.

Species Tree analysis was done using BEAST2 v.2.4.5 (Bouckaert et al., 2014), carried out using the StarBEAST 2.5 template (Heled and Drummond, 2010). Contrasting with the concatenated dataset, in the Species Tree analyses only closely related species of *Astyanax* were included as an outgroup. Tree and clock models were configured linking mitochondrial (*COI* and *ND2*) loci, and considering each nuclear gene (*RAG2, MYH6*, and *S7*) unlinked. Morphologically delimited species were used as terminals as criteria for grouping specimens into putative species. Multi-species coalescence prior was set to linear with constant root; and a tree model set to the birth-death model with uniform distribution. First, the Species Tree was generated without prior calibrations for date estimates on nodes, and later a Species Tree was generated that includes prior calibration dates on nodes based on divergence times estimation from the concatenated dataset (see divergence time estimates analysis below). Priors for divergence time estimates were used on nodes under a normal distribution and were restricted to those nodes were the concatenated time estimated tree was congruent with the first Species Tree analyses (early divergence nodes in *Oligosarcus*). Three separate runs for the Species Tree analyses were run to check for convergence in the topologies, using 200 million generations, logging every 20 million generations to yield a posterior distribution of 10.000 topologies. Inspection for stationary posterior probabilities of all parameters was done using Tracer v1.6 (Rambaut et al., 2014). Convergence established by Effective Sample Size (ESS) of parameters above 200. Ten percent of the trees were discarded as burn-in. The remaining trees were used to compute a summary tree using the maximum clade credibility tree function with TreeAnnotator 2.4.3 (Bouckaert et al., 2014). All these analyses were implemented by XSEDE (3.2.6) in the CIPRES portal (Miller et al., 2010).

2.4. *Molecular clocks and divergence time estimation*

Gene sequences were subjected to a molecular time divergence analysis in BEAST v.2.5.1 (Bouckaert et al., 2014), using all taxa included in Table S1 and rooting in *Serrasalmus* sp. For that, absolute node age for three fossils was used as calibration points, following the assumptions of Lemey and Posada (2009), Parham et al. (2012) and Heath et al. (2014).

†*Paleotetra* spp., dated to Eocene-Oligocene, were collected in Entre-córregos formation, Aiuruoca basin, Minas Gerais, Brazil (Weiss *et al*., 2012), and represents a stem Characidae according to a recent total-evidence analysis of Characiformes (Mirande, 2018). This fossil is included in the present study to help constrain the minimum age of the basal node formed by Characidae + Triportheidae + Gasteropelecidae

clade. †*Lignobrycon ligniticus* (Woodward, 1898), dated to Late Oligocene, belongs to Triportheidae and was collected in the Tremembé Formation, Taubaté Basin, São Paulo, Brazil (Malabarba, 1998). This fossil is used to constrain the minimum age for the node of Triportheidae species. †*Megacheirodon unicus* (Travassos and Santos, 1955), also dated to Late Oligocene, was also collected in the Tremembé Formation (Malabarba, 1998), and was used to date the minimum age of the node subtending Cheirodontinae species.

Estimated dates used as lognormal priors in BEAST2 were implemented as minimum age offsets for †*Paleotetra* spp. (33.9 Ma – Eocene, with 1.0 of Mean and standard deviation), and †*L. ligniticus* and †*M. unicus* (23.03 Ma – Oligocene, with 1.3 of Mean and standard deviation), according to the minimum age of these time periods determined by the International Commission on Stratigraphy - FICS – (www.stratigraphy.org). A relaxed lognormal clock model was set and a Fossilized Birth-Death model was used as a tree prior (Heath et al., 2014). The analysis was performed with 400 million generations with sampled trees every 40 million generations. Stationarity and sufficient mixing of parameters (ESS > 200) were checked using Tracer 1.6.

Finally, in addition to the three fossil calibration points mentioned above, another two analyses were generated with the inclusion of an *Oligosarcus* fossil (Bogan & Reyes, 2009). This is a fossil (a dentary) of †*Oligosarcus* sp. from the Centinela del Mar formation (Buenos Aires, Argentina) from the late Pleistocene (230-125 Ka; according to Bogan & Reyes, 2009). The identification of this fossil is currently difficult due to a lack of autapomorphic characters, but this fossil share tooth patterns with some extant species occurring in the region like *O. jenynsii* and *O. oligolepis*. Therefore, we created two node-dating scenarios: (1) placing the fossil at the base of the node with *O. oligolepis* + *O. robustus*, and (2) at the basal node of *O. jenynsii* and its closely related species (*O. jacuiensis*, *O. brevioris*, *O. bolivianus*, and *O. varii*). Results of these calibrated trees using †*Oligosarcus* sp. were included in the supplementary material 2.

2.5. *Ancestral range estimation*

We use event-based analyses to evaluate biogeographical processes delineating *Oligosarcus* species distribution and putative cases of allopatric speciation (vicariance), allopatric with secondary contact (dispersal) and sympatric speciation (within-area speciation). We constructed a taxon-area matrix of *Oligosarcus* species distributions

using geographic operational units. Geographic unit delimitation is similar to the Freshwater Ecoregions of the World (FEOW) proposed by Abell et al. (2008), with exception of joining of Lower and Upper Uruguay FEOW's and coastal FEOW's in eastern Brazil into South, Central, and North Coastal geographical units, following species distribution limits in this area. FEOW ecoregions have been used as operational geographic units in biographical studies of aquatic fauna either explicitly (*e.g.* Albert and Carvalho, 2011) or in similar delineations (*e.g.* Tagliacollo et al., 2015; Machado et al., 2018). A total of nine geographical units were used in the analyses of geographic distribution (Fig. 2), with six inland areas draining to the La Plata Basin: Chaco (CB), Paraguay (PA), Upper Paraná (UP), Lower Paraná (LP), Iguaçu (IG), and Uruguay (UR; correspond to both Upper and Lower Uruguay ecoregions); and three coastal drainage areas: North Coastal (NC; corresponding to Northeastern Mata Atlântica ecoregion), Central Coastal (CC; including Paraíba do Sul, Fluminense, Ribeira de Iguape and Southeastern Mata Atlântica ecoregions), and South Coastal (SC- including Laguna dos Patos and Tramandaí-Mampituba ecoregions). Presence/absence of species within the operational geographic units were coded based on distributional data from Ribeiro and Menezes (2015: figs. 17-18) and our additional records (Table S4).

FIGURE 2.

We evaluated Landscape Evolution Models (LEMs) using the information on connectivity between these areas according to important geographic events occurring southeastern South America. We did that by changing dispersal matrices to correspond to connection and isolation events of the geographically adjacent basins in three distinct time frames. Additionally, a "null model" (M0) was generated, without considering the influence of any geographic event on the ancestral range estimation of *Oligosarcus*. Three alternative scenarios (LEMs) for the range evolution in *Oligosarcus* were designed, and each LEM have three periods: 1) 5-2.8 Ma, 2) 2.8-2.0 Ma and 3) 2.0-present. The oldest time considered in the geographic analysis was based on the estimated maximum age for the genus *Oligosarcus* (recovered with divergence time analyzes), followed by the beginning of the Pleistocene, and the estimated age of formation of the Iguaçu and Sete-Quedas waterfalls (see below for reasoning). All models (M0 and LEMs 1-3) were tested using two model-based analytical methods in historical biogeography (*e.g.* DIVALIKE and DEC, respectively Ronquist, 1997; Ree and Smith, 2008). These analytical methods estimate ancestral ranges based on relevant biogeographical parameters including,

dispersal and range contraction using the package BioGeoBEARS in R (Matzke, 2013, 2014).

In our analyses, we evaluate only models that accommodate vicariance (*e.g.* DEC and DIVA), since these models include most biogeographical events associated with the diversification of *Oligosarcus* across drainage basins of southeastern South America (*e.g.* Menezes, 1988; Ribeiro, 2006; Machado et al., 2018). We avoid using the founder-event speciation parameter +J based on recent criticism (Ree and Sanmartín, 2018). The maximum number of areas occupied by a lineage was set to six, which is more than the maximum number of areas currently observed in any of the species analyzed (*e.g. O. jenynsii* occurs in three areas). An ultrametric phylogeny obtained with age constrained Species Tree analyses (see above) was used for the biogeographic analysis.

Landscape Evolution Models were designed considering two geographic events: (1) the connection between coastal drainages starting at the Pleistocene (2.8 Ma) through successive periods of marine regression, which may have favored freshwater species dispersal throughout this region in a stepping stone manner, and (2) the isolation of the Iguaçu and Upper Paraná basins of the other drainages of La Plata (continental region) through formation of Iguaçu and Sete-Quedas waterfalls in the late Pleistocene (Stevaux, 1994), which may have isolated lineages in these two regions. The connection event during the Pleistocene is an essential event in the evolutionary history of many fish species (Weitzman et al., 1988), being explicitly tested in several works (Thomaz et al., 2015b, 2017). In the same way, the isolation events of the Iguaçu and upper Paraná basins through a process of headwater erosion (Stevaux and Latrubesse, 2010) represents an important role in the isolation of several lineages of freshwater fishes (Zawadzki et al., 1999; Prioli et al., 2002; Souza-Shibatta et al., 2018), corroborating a high degree of endemism in the Iguaçu and Upper Paraná river basins (Abell et al., 2008; Baumgartner et al., 2012).

The different scenarios are illustrated in Figure 3. To test these different scenarios (LEMs), matrices were constructed: (1) adjacent-area matrix (informing which areas are adjacent to each other, Table S5), (2) time-period matrix (informing the different time frames which each event is contributing), and (3) manual dispersal multipliers constraining or allowing dispersal. More specifically these models are relaxing dispersal between coastal drainages after the beginning of the Pleistocene – 2.8 Ma; LEM 1), imposing dispersal restriction to Iguaçu and Upper Paraná after establishment of Iguaçu and Sete Quedas falls at approximately 2 Ma ago; LEM 2), and considering both

geographical events mentioned above (LEM 3; see Tables S6-S11). However, because events of river capture and dispersal may have occurred after the formation of barriers, none of the models considered total impermeability for dispersal rates. Therefore, two different dispersal rates values were examined (quasi-impermeable – rate 0.1 and semi-permeable – rate 0.5) in order to observe possible differences in model choice and the range reconstructions (Table 1).

FIGURE 3.

3. Results

Two mitochondrial (*COI* and *ND2*) and three nuclear (*RAG2*, *MYH6*, and *S7*) markers were sequenced, resulting in a total concatenated alignment of 4,321 base pairs (1,720 in the mitochondrial partition and 2,601 in the nuclear partition). Of these, *S7* had 129 variable sites, while *MYH6* had 233 and *RAG2* had 460 variable sites, these last two genes encompass a more substantial taxonomic diversity. Between mitochondrial genes, *ND2* was the most variable marker (Table S12). For the joint mitochondrial and nuclear analysis, 1,797 sites were variable. Best-fit models of nucleotide substitution have nine partitions for the concatenated MrBayes and dating analysis in BEAST, and five partitions for Species Tree analysis (Table S3).

3.1 Phylogenetic reconstruction of Oligosarcus and related taxa

Oligosarcus was resolved as a monophyletic group, with high posterior probability (PP = 1.0) in all analyses (Figs. 4-6); is composed by two monophyletic groups (herein called Coastal and Continental Group). In addition, all analyzes support *Oligosarcus* as closely related to a clade of species of *Astyanax sensu lato*, with high posterior probability (≥ 0.96), and both are forming a clade sister to *Astyanax* clade *sensu* Mirande (2018), with high posterior probabilities (≥ 0.95) (Figs. 4-5).

FIGURE 4.

The concatenated (Fig. 4) and Species Tree (Fig. 5) analyses (using Bayesian Inference) resulted in largely congruent topologies. Both analyses produced phylogenies with strong to moderate posterior probability (strong ≥ 0.95; moderate 0.80-0.94) for basal nodes within *Oligosarcus*. The phylogenetic analysis using Maximum Likelihood recovered the same topology as found in the concatenated Bayesian analysis, and is therefore presented only as supplementary material (Fig. S6).

The Continental Group was composed by species distributed mostly in the La Plata River basin, but also in the Laguna dos Patos and in the Tramandaí River systems.

In the concatenated analysis, *Oligosarcus longirostris* is the sister species of two clades including the remaining species of Continental Group. One clade is composed by species from the Upper Paraná and Paraguay rivers, where *O. planaltinae* is the sister of *O. paranensis* and both form a clade along with *O. pintoi*, a sister group of *O. perdido*, all these clades have high posterior probabilities (Fig. 4). On the other hand, another large clade within Continental Group has relatively short branches and low posterior probability for their species relationships; this includes *O. brevioris*, *O. varii*, *O. bolivianus*, *O. jacuiensis* and *O. jenynsii*. In the Species Tree analysis, Continental Group has the same composition, but with slightly different relationships when compared with the concatenated analysis. *Oligosarcus planaltinae* is the sister of the clade (*O. paranensis* + *O. pintoi*) and *O. perdido* is the sister species of the clade (*O. brevioris* + (*O. varii* + (*O. bolivianus* + (*O. jacuiensis* + *O. jenynsii*)))). The Species Tree analysis, in general, has higher support values for the species relationships of Continental Group (Fig. 5). Coastal Group is composed of species distributed within coastal drainages of southern and eastern Brazil, except for *O. oligolepis* that is found in the Lower Uruguay and Lower Paraná rivers. In the concatenated analysis, *O. hepsetus* population from the Paraíba do Sul River was found as sister species to remaining species of Coastal Group and these species forming two distinct groups. A clade formed by species from Doce and Jequitinhonha river basins was found, where *O. macrolepis* is the sister species of a clade composed by *O. argenteus* and *O. solitarius*, these relationships were found in both concatenated and Species Tree analyses, with high posterior probabilities.

In the concatenated analysis, a lineage of *Oligosarcus hepsetus* (sampled in small coastal rivers draining the Rio de Janeiro and Espírito Santo states) is the sister of *O. acutirostris*, and the third lineage of *O. hepsetus* (sampled in the Ribeira de Iguape and Itanhaém river basins in the São Paulo State) is sister to a clade composed by the southern species *O. robustus* and *O. oligolepis* (Fig. 4). In the Species Tree analysis, *O. hepsetus* was observed as sister species of *O. acutirostris*, with relatively low node support (PP=0.61, see Fig. 5).

Within these two major *Oligosarcus* groups, when gene trees were analyzed separately (Figs. S1-S5), some differences between markers were found. One main difference is that the nuclear markers found the species of the Upper Paraná and Paraguay rivers as a monophyletic group, in contrast to the mitochondrial tree that suggests different topologies (Figs. S1-S2). A population, tentatively identified as *O. hepsetus*, from Sombrio Lagoon in southern Santa Catarina State in the Tramandaí-Mampituba

ecoregion was included in the Continental Group in the mitochondrial gene trees (Fig. S1) and in the Coastal Group in the nuclear genes (Fig. S3-S5) for this reason this population was removed from the concatenated and species tree analyses.

3.2. *Species monophyly*

Most species of *Oligosarcus* were found as monophyletic units. However, we have found instances of species polyphyly or paraphyly when examining separated gene trees or the concatenated dataset. Within Coastal Group, specimens morphologically identified as *O. hepsetus* and collected on its distribution area were found in three distinct clades (see Fig. 4). Also, within Coastal Group, *O. solitarius* is found nested within *O. argenteus* samples in both gene trees and also in the concatenated dataset (Figs. 4 and S3). Similarly, within Continental Group, the relationships recovered by concatenated data showed *O. jenynsii* as polyphyletic with *O. bolivianus*, *O. jacuiensis*, *O. brevioris* and *O. varii* specimens nested within this species (Fig. 4). *Oligosarcus brevioris* was not resolved as monophyletic, with *O. varii* nested within populations of this former species in the concatenated data set (Fig. 4) and gene trees varying regarding this matter.

FIGURE 5.

3.3 Divergence time estimation

The dated phylogenetic reconstruction (Fig. 6) estimated that the origin of *Oligosarcus* radiation was in the Pliocene around 4.13 Ma (±5.75-2.87 Ma), and range estimation varied between late Miocene and Pleistocene. Early branching events for major *Oligosarcus* lineages were estimated to occur within the Pleistocene around 2.84 Ma (±4.11-1.71 Ma) for the Continental Group, and 2.95 Ma (±4.08-1.99 Ma) for the Coastal Group. Most species were estimated to have diverged within the Pleistocene with average estimates that vary between 1.8 to 0.2 Ma. Estimated average ages of divergence between *O. robustus* and *O. oligolepis* or the radiation of *O. jenynsii* and its closest relative do not reject the minimum ages supported of these groups as previously indicated by the fossil unidentified of *Oligosarcus* sp. from the Pleistocene in the Centinela del Mar formation in Argentina. Inclusion of this fossil does not strongly influence age estimation in *Oligosarcus* (Fig. S9-S10; see Material and Methods). The age estimates for the remaining clades (outside *Oligosarcus*) recovered the crown group Characidae radiation to around Middle Eocene at 43.91 Ma (±51.84-35.75 Ma). The clade composed by Cheirodontinae, Characinae, and Tetragonopterinae species has age estimates of 35.50

Ma (±46.17-32.12 Ma), and Stevardiinae 24.11 Ma (±33.54-17.39 Ma) for the average age of their radiation, which corresponded to ages between the Oligocene and Miocene.

FIGURE 6.

3.4 Ancestral range estimation

The ancestral range estimation demonstrated that DIVALIKE model was the best fitting model for all the landscape evolution reconstructions (Table 1). When DEC and DIVALIKE models are compared for each of the evaluated landscape models, the analyses showed that DIVALIKE presented the highest likelihood and lowest AICc values (Table 1). When comparing LEMs, we observed that the most likely scenario was LEM 3 (DIVALIKE), when dispersal constrictions and relaxation between the La Plata and Coastal areas were included in the model, this considering both quasi-impermeable and semi-permeable dispersal rates (0.1 and 0.5; Table 1). The best model of ancestral range estimates is pictured in Figure 7, and other models are presented in the supplementary material 2 (Figs. S11-S12).

The model-based analyses estimated an early vicariant event between two large groups of *Oligosarcus* species inhabiting coastal and continental basins, that occurred at approximately 4.0 Ma (Fig. 7). One group was restricted to the highlands of the La Plata Region (Upper Paraná - Continental Group), and another group restricted to the Central and Northern Coastal areas (Coastal Group).

Within the Continental Group, species distributions are mostly in inland areas such as the Chaco, Paraguay, Upper/Lower Paraná, Iguaçu, and Uruguay basins, although some species in this group also inhabit the Laguna dos Patos and Tramandaí-Mampituba basins in the South Coastal geographic area. This group seems to have evolved within upland areas, and then later dispersed to the lowlands (Fig. 7). The ancestral lineage within the Continental Group occupied the Upper Paraná area and then expanded its range to include the Iguaçu area. Then a vicariant event at about 2.84 Ma isolated these areas, contributing to the first cladogenetic event within the Continental Group (Fig. 7), separating the Iguaçu and Upper Paraná lineages. Species occurring in the Upper Paraná expanded their occurrence area to the Lower Paraná and underwent another vicariant event that isolated lineages in the Upper Paraná (*O. paranensis*, *O. pintoi* and *O. planaltinae*) from the lower Paraná basins. Evolution within the Continental Group was then followed by two important dispersal/vicariant events, which isolated the Paraguay and Chaco ecoregion. Lineage evolution within the Continental Group shows vicariant

events separating the lineages of the Iguaçu and Upper Paraná from the remaining basins. After that, species with occurrence in La Plata drainage went through a range expansion into adjacent areas such as Uruguay, Laguna dos Patos and Tramandaí-Mampituba basins, followed by vicariant events, separating these lineages resulting in several endemic species (Fig. 7).

Within the Coastal Group, species are mainly restricted to coastal areas, but at least one species is present in Lower Parana and Uruguay areas, which is the result of a recent range expansion to this region (Fig. 7). This group evolved in the Central and North Coastal areas and later went through a vicariant process (Figs. 7). According to the DIVALIKE on the LEM3 scenario, the clade composed by *O. macrolepis*, *O. argenteus* and *O. solitarius* species evolved primarily from a vicariant process, which separated Central and North Coastal areas, restricting this clade in the North Coastal area and upland regions approximately in 2.4 Ma ago.

The clade that is restricted to the Central Coastal area (after its isolation from the North Coastal) expanded its occurrence to the South Coastal area, and then went through a vicariant process again, isolating both areas (Central and South). A new northward expansion process occurs from the Central Coastal area to the North Coastal area, which then undergoes a vicariant process isolating the lineages *O. acutirostris* + *O. hepsetus*. A lineage (*O. robustus* + *O. oligolepis*) that was restricted to South Coastal expanded its range and underwent a vicariant event that separated the coastal drainages (Laguna das Patos and Tramandaí-Mampituba) from continental basins (Uruguay and Lower Paraná).

TABLE 1.

FIGURE 7.

4. Discussion

4.1 Phylogenetics of Oligosarcus and related groups

Our comprehensive phylogeny of *Oligosarcus* species, based on a multilocus dataset, found strong support for the monophyly of *Oligosarcus* corroborating other authors that used morphological data (Mirande 2010; Mirande et al., 2011; Ribeiro and Menezes, 2015) or combined evidence (Mirande, 2018). Unfortunately, it was not possible to assess the phylogenetic position of disputed *Oligosarcus* species such as *O. itau*, *O. amome* and *O. platensis* that are known from only a few specimens within their type series, and for which no genetic data are available (Mirande et al., 2011; Almirón et al., 2015). The major discrepancy between our results and those of previous studies regards the intrageneric relationships of *Oligosarcus*. In our study, the first split in

Oligosarcus was between two lineages with somewhat equivalent species diversity, different from the results obtained using morphological data (Ribeiro and Menezes, 2015), where *O. pintoi* is sister to remaining species of the genus.

Ribeiro and Menezes (2015) found *O. pintoi* as sister of the remaining species of the genus based on putatively plesiomorphic features of teeth morphology (tricuspid in *O. pintoi* vs. pentacuspid in other species studied by the authors). In our study, *O. pintoi* is nested within a small group, which may indicate the reversal of teeth morphology in this species. Interesting to note that a more inclusive position for *O. pintoi* is also found by others phylogenies using morphology (Mirande et al., 2011; Almirón et al., 2015) and combined evidence analysis (Mirande, 2018), which suggests that morphological data may support the position of *O. pintoi* among a crown group of *Oligosarcus*. Morphological features such as the number of teeth in the maxilla, presence of slightly developed foramen in the premaxilla, and the relative positions of ectopterygoid and dentary teeth, compose some of the characters that support different clades in phylogenetic results proposed by Ribeiro and Menezes (2015). Disagreements on the relationships showed by both sets of data (molecular and morphological) may reflect adaptive convergence associated with diets (*e.g*. piscivory vs. omnivory) and/or habitats (*e.g*. lacustrine vs. riverine). These types of convergent adaptations are quite frequent in fishes, including several examples in Characidae (Ornelas-Garcia et al., 2008; Kowalko et al., 2013; Silva-Camacho et al., 2014; Aguilar-Betancourt et al., 2017; Roxo et al., 2017; Kolmann et al., 2018).

As an example, Garita-Alvarado et al. (2018) examined morphological diversity of *Astyanax* from Central America (including "*Bramocharax*") and tested the influences of the environment (riverine and lacustrine) on the diversification of the group, proving the occurrence of convergent evolution, which led to the previous classification of "*Bramocharax*" as a different genus (Rosen, 1972; Lima et al., 2003). Similarly, phenotypic divergence in *Oligosarcus* may also be observed as a consequence of habitat use and diet. In *Oligosarcus*, we found species with distinct body shapes (*e.g*. premaxilla with or without foramen, snout length, body height, number of maxillary teeth) as closely related (*e.g. O. argenteus* and *O. solitarius*), contrasting the morphological phylogeny, which hypothesized these species in distinct clades (Ribeiro and Menezes, 2015). In this example, *O. argenteus* is restricted to riverine habitats, whereas *O. solitarius* is restricted to lakes in the middle Doce basin (Barros et al., 2015) and these environments can restrict the body size. Another example is the distinct morphology of *O. pintoi* that diverged in

sympatry (speciation within-area; see ancestral range estimation Fig. 7) from a common ancestor with *O. paranensis* and both possess quite distinct mouth and tooth morphologies, which may be associated with their different diets (Casatti, 2003).

Therefore, convergent and parallel evolution can lead to incongruent hypothesis between molecular and morphological phylogenies (Zakon, 2002; Wiens et al., 2003; Woodard et al., 2011; Parker et al., 2013). Also, other biological processes may also significantly influence phylogenetic results, such as introgression, incomplete lineage sorting and hybridization, leading to taxonomic incongruences between morphological and molecular data (Mutanen et al., 2016; Bravo et al., 2019). Regarding the non-monophyletic species examined here, these biological processes also can be related to the difficulty of identifying and defining species and more likely can occur among recently diverged species than older lineages (Mutanen et al., 2016). In our study, three species (*O. hepsetus, O. jenynsii,* and *O. brevioris*) were observed as polyphyletic and one species as paraphyletic (*O. argenteus* concerning *O. solitarius*). These cases seem to reflect recent divergence estimates of speciation dates within this genus radiation. Instances of hybridization in *Oligosarcus* are a serious problem in species delimitation (Aguiar, 2011), and may in some cases impede the speciation processes (Abbott et al., 2013). In the present work we observed a putative population of *O. hepsetus* collected in Sombrio lagoon near the geographical limit of *O. jenynsii* and *O. hepsetus,* which may represent a case of genetic introgression between species of the deeply-diverged Continental and Coastal groups, as evidenced by different results in genes trees recovered using nuclear and mitochondrial markers (Figs. S1-S5).

4.2 Time divergences and historical biogeography

The origin and diversification of *Oligosarcus* in the Pliocene, and cladogenetic events within the group, are somewhat different from previous estimated dates based on phylogenetic relationships of morphological data only, inferred from the allopatric distributions of species and hypothesized biogeographical events during the Miocene, approximately 15 Ma ago (McQuarrie et al., 2005). Ribeiro and Menezes (2015) observed a clade composed of the Andean species *O. schindleri* and *O. bolivianus*, and hypothesized a cladogenetic event related to the rise of the Andean Trust Belt that caused the change in the course of the rivers within this region and putatively isolated these lineages. Although *O. schindleri* is not included in this analysis, the minimal time divergence estimation proposed by Ribeiro and Menezes (2015) for the genus is unlikely

574 based on results of our time-calibrated tree: 1) *O. bolivianus* is well nested within the
575 Continental Group with much younger age estimates (Pleistocene), and 2) the entire
576 *Oligosarcus* radiation is estimated around 4.13 Ma (±5.75 to 2.87 Ma). If *O. schindleri* is
577 the sister species to *O. bolivianus* (Menezes, 1988; Ribeiro and Menezes, 2015), the
578 divergence between these lineages is probably more recent than that proposed by Ribeiro
579 and Menezes (2015). For example, there is evidence for more recent hydrographic
580 exchanges among drainages in the western portion of Andes across the watershed of the
581 Paraguay and Upper Madeira basins, which may have promoted species dispersal
582 (Carvalho and Albert, 2011). Faunal exchange events, such as those promoted by the
583 megafan river behavior on Chaco (*e.g.* Río Grande–Parapetí/Pilcomayo) date as recent as
584 35–1.4 Ka and may have supported a more recently dispersion of *O. schindleri* and *O.*
585 *bolivianus* within these drainages (Wilkinson et al., 2006).
586 Regarding the ancestral range of *Oligosarcus*, it has been proposed that its
587 ancestral species inhabited uplands of the Brazilian Shield and later dispersed to rivers in
588 the lowland regions of South America (Menezes, 1988; Ribeiro and Menezes, 2015). As
589 observed by the analyses of ancestral range estimation, and as proposed by Menezes
590 (1988), an area in the region of the Brazilian Shield (*e.g*. Upper Paraná, Central and North
591 coastal basins) was estimated as the ancestral area of the genus. Although our analysis
592 supports this interpretation of dispersal of *Oligosarcus* lineages to the lowlands (Fig. 7;
593 taxa inside black squares), we observed two independent events, one in the Continental
594 group and another in the Coastal group.
595 It has been proposed that during the Tertiary (Cenozoic), and associated with
596 neotectonic events, there were points of ichthyofaunal interchange among the shield
597 rivers draining to the coastal and interior basins (Ribeiro, 2006). Our analysis indicates
598 the radiation of each one of the larger *Oligosarcus* lineages is almost restricted to La Plata
599 basin and its tributaries (Continental Group), and another occurring in the coastal region
600 of Brazil (Coastal Group). Exchanges between coastal and inland basins seem to be rare
601 within *Oligosarcus* radiation and limited to the region in its southernmost distribution
602 limit between Uruguay and Laguna dos Patos, and northern limit to São Francisco and
603 Doce rivers (for *O. argenteus*). Although populations of *O. argenteus* from São Francisco
604 River were not evaluated in our study, Barros et al. (2015) reported a questionably
605 conspecific population of *O. argenteus* occurring in the São Francisco (Continental) and
606 Doce (Coastal) rivers, that diverge in terms of genetic, karyotype and morphological

differences, representing a classic example of communication between coastal and continental drainages within the Brazilian crystalline shield.

In our analyses, the DIVALIKE model estimated an early vicariant event followed by several range expansions, which in turn were followed by vicariance, resulting in the current distribution of the genus. This history may be associated with the allopatric pattern observed among most species of *Oligosarcus*. Menezes (1988), although not performing a phylogenetic analysis, proposed that vicariance was the main process of diversification within the genus, justified by the distribution patterns of species and its restriction to areas of endemism in South America.

The diversification of the continental group within the La Plata River basin is marked by the appearance of geographic barriers that resulted in the isolation of *O. longirostris* in the Iguaçu and the ancestor of *O. paranensis*, *O. pintoi* and *O. planaltinae* in the Upper Paraná during the Pliocene and Pleistocene. The formation of the waterfall barriers of Iguaçu and Sete-Quedas that separate the modern Iguaçu and Upper Paraná basins from the rest of the Paraná system may be linked with these processes as suggested by the LEM 3. These waterfall barriers have been hypothesized to be responsible for the high endemism of these two basins (Baumgartner et al., 2012; Langeani et al., 2007). The Iguaçu falls has been moving upstream by headward erosion during the Pleistocene between 1.5 to 2.0 Ma (Stevaux and Latrubesse, 2010). The period of formation of the Sete-Quedas falls is uncertain, but may have been concomitant with that of the Iguaçu falls during the Quaternary (Stevaux, 1994; Orfeo and Stevaux, 2002). In general, these geological dates are congruent with the model fitting analyses proposed in this study. One exception to this pattern of endemism in the upper Paraná is *O. pintoi*, which is also known from parts of the upper Paraguay and upper Guaporé river basins (Ribeiro and Menezes, 2015). The presence of *O. pintoi* in upper Paraguay may be the result of a relatively recent dispersal events (<1.0 Ma) contrasting with a relatively older proposed dates associated with tectonic events that, according Ribeiro and Menezes (2015), reactivated ancient fault zones of the Precambrian central Brazilian region (2.5 Ma), and promoted the dispersal of *O. pintoi*.

In addition to the numerous allopatric species of *Oligosarcus*, several sympatric speciation events may also have occurred. For example, the separation of *O. pintoi* from *O. paranensis* seems to be the result of sympatric speciation event in the Upper Paraná basin to at least 980 Ka (Fig. 3 and 6). Sympatric speciation is always difficult to assess and often associated with niche partition and disruptive selection (Seehausen and Wagner,

2014). The other lineage of the Continental Group, ancestor to *O. bolivianus*, *O. jenynsii*, *O. jacuiensis* and *O. brevioris*, dispersed via tributaries of the La Plata River basin, subsequently becoming isolated and diverging into new species.

On the other hand, the species of Coastal Group have an extensive distribution through the isolated drainages within the coastal region of Brazil. This region has a lower diversity when compared to other areas, but a high endemism (Weitzman et al., 1988). Menezes (1987, 1988) observed this pattern of coastal endemism for *Oligosarcus* and Weitzman et al. (1988) for *Mimagoniates*. Several studies have sought to understand distribution patterns of obligate freshwater fishes in coastal regions of southeastern Brazil (Hirshmann et al., 2015; Thomaz et al., 2015b, 2017). One of the main mechanisms hypothesized for dispersal and isolation is Pleistocene sea-level fluctuations (Thomaz et al., 2015b, 2017; Thomaz & Knowles, 2018). The idea is that freshwater coastal fish species disperse and occupy extensive areas of the coastal plain during low sea-level stands, and then becoming isolated when sea-levels rise (Buckup, 2011). These cyclic shoreline advances and retreats repeatedly severed and reestablished gene flow among populations in different coastal sub-basins, resulting in higher rates of both speciation and extinction (Albert et al., 2011).

Range extension of *Oligosarcus* lineages in the Coastal Group seems to be temporally and mechanistically associated with the sea-level changes in this region. This is supported by examining relaxation in the dispersal matrices probabilities between these areas in the LEM 3. Therefore, these repeated events of sea-level rise and retreat might have promoted range extension towards both north and southward portions of the coastal region. Interestingly, although many lineages seem to have used this coastal pathway for expanding their ranges, one clade composed by *O. argenteus*, *O. solitarius* and *O. macrolepis* remained restricted to the North Coastal area. The contrasting ranges of these lineages may indicate that the connections between coastal areas may be rather filters for dispersal and only some freshwater fishes in this region used these dispersal routes. Their vagility may be associated with their habitat preferences such as lowlands and highlands, fostering highland, likely less influenced by paleodrainage connection to be confined to a single basin in the North Coastal area (*e.g*. restriction of the occurrence of *O. argenteus* and its relatives *O. solitarius* and *O. macrolepis* in the coastal region).

5. Conclusions

Using a multilocus dataset, we present a hypothesis of interspecific relationships among *Oligosarcus* species and the phylogenetic position of *Oligosarcus* among closely related clades of Characidae. Besides, we present an estimation of lineage divergence times for the group, as well as biogeographical ancestral range estimations. The phylogeny presented substantially expands understanding of the relationships, character evolution and biogeographic history of *Oligosarcus*, and gives insights into the diversification of the group, as well as a greater understanding about diversification of Neotropical fishes and the related processes. Finally, our study supports the importance in using the geological information in the construction of Landscape Evolution Models as an analytical method in historical biogeography (Smith 2009; Buerki et al., 2011) as previously supported by other studies using freshwater fishes (Bossu et al., 2013; Tagliacollo et al., 2017; 2015; Machado et al., 2018).

Acknowledgments

We thank the curators of the following ichthyological collections and personal for sending material: F. Langeani (DZSJRP); C. Oliveira (LBP); C. Lucena (MCP); F. Jerep and O. Shibatta (MZUEL); and F. Carvalho (ZUFMS). Thanks to J. Wingert to help assessing specimens at UFRGS fish collection. Thanks to T. Paim for all the help and technical support in the molecular laboratory of the Department of Zoology. We also thank J. Dergam for sending a complete sequence of the ND2 gene from a specimen of *O. argenteus*, which made it possible to design the primers used in present study. We thank for help and comments on the biogeographical analyses A. Ferrari, N. Matzke, V. Tagliacollo, and A. Thomaz. An early version of this manuscript received criticism and comments from J. Albert, A. Thomaz, B. Calegari, A. Ferrari and A. Netto-Ferreira, which improved its final version. Thanks to M. Mirande for sending *COI* sequences of *O. bolivianus*. For helping collecting specimens of *Oligosarcus* in field expeditions we thank Fernando Carvalho, Maria José Alencar Vilela, Laura Modesti Donin, Iago Penido, Tiago Debona, Juliano Ferrer, Amanda Santos, Dario Faustino, Vanessa Meza, Matheus Gallas Lopes, Karine Bonatto, Rafael Angrizani, Gabriele Volkmer and Yan Soares. Collections of *O. perdido* were made with help of F. Carvalho and its funded project (Fundect #59/300.093/2017, SIAFEM 27248). This work was partially funded by a CNPq Universal grant (401204/2016-2) to LRM. TPC is funded by CAPES with a PNPD postdoctoral fellowship, EWW is funded by CAPES with a doctorate scholarship and by

Systematics Research Fund 2017/18, and PCS was funded by CNPq with a PDJ postdoctoral fellowship (150956/2017-7).

Appendix A. Supplementary material

The following are the Supplementary data to this article:

Supplementary 1 - Tables S1–S12

Supplementary 2 - Figures S1–S12

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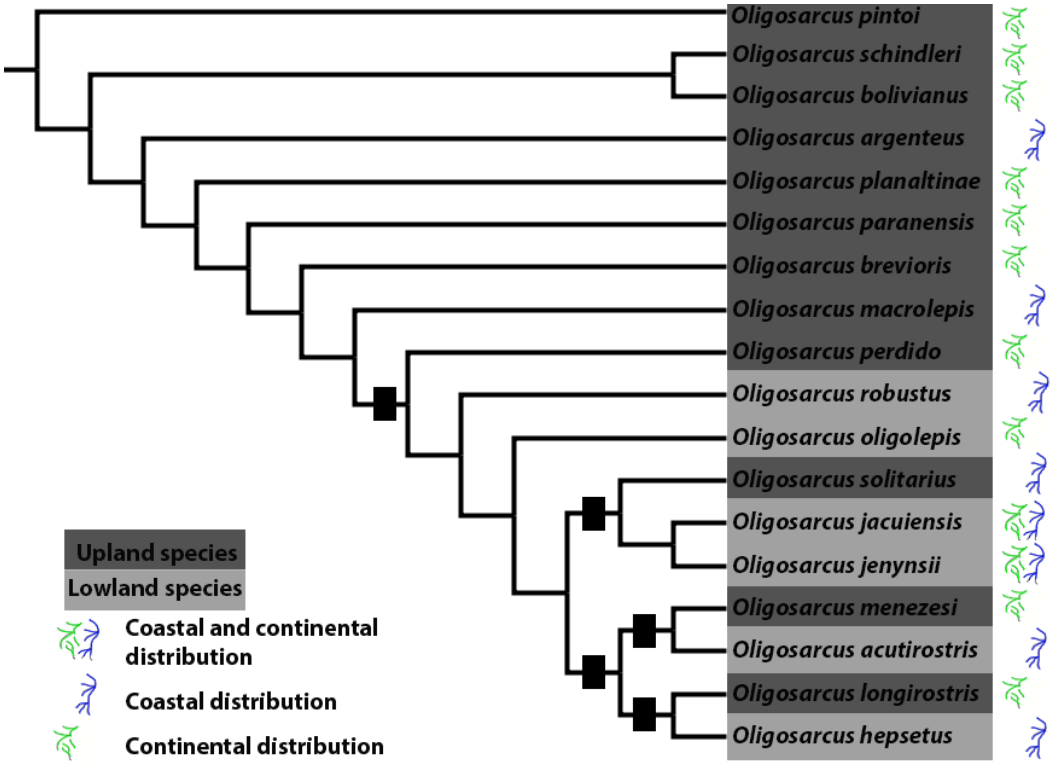
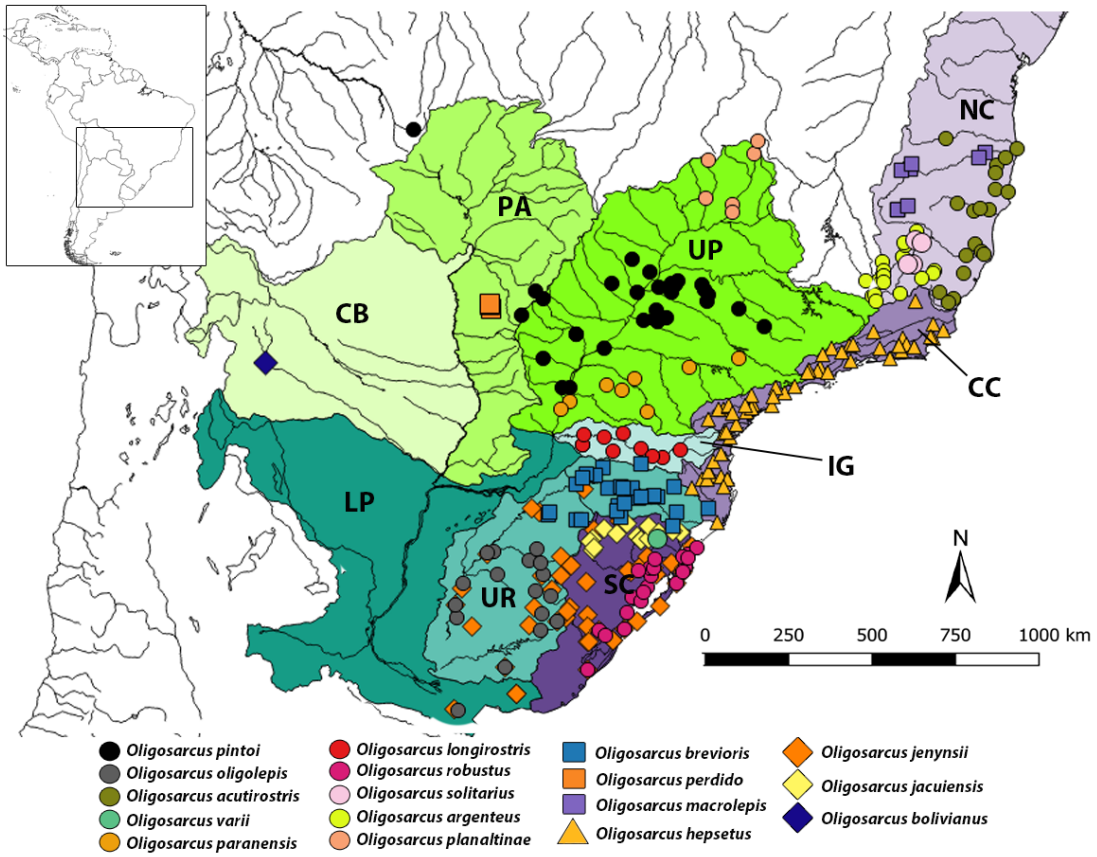


Figure 1. Previously proposed interspecific relationships of *Oligosarcus* species based on a parsimony analysis of 34 morphological characters (imaged modified from Ribeiro and Menezes, 2015: fig.1 9). Species distribution on lowland (light gray) and upland (dark grey) river basins. Black squares at the base of nodes represent putative vicariant events between lowland and upland distributed taxa after range expansion (Ribeiro and Menezes, 2015). Geographic distributions of each *Oligosarcus* species in coastal and continental river basins are marked in the right side of the species names.



1158 Figure 2. Map illustrating the nine geographic areas used in the biogeographical analysis and distribution
1159 of *Oligosarcus* species included in the phylogenetic analyses.

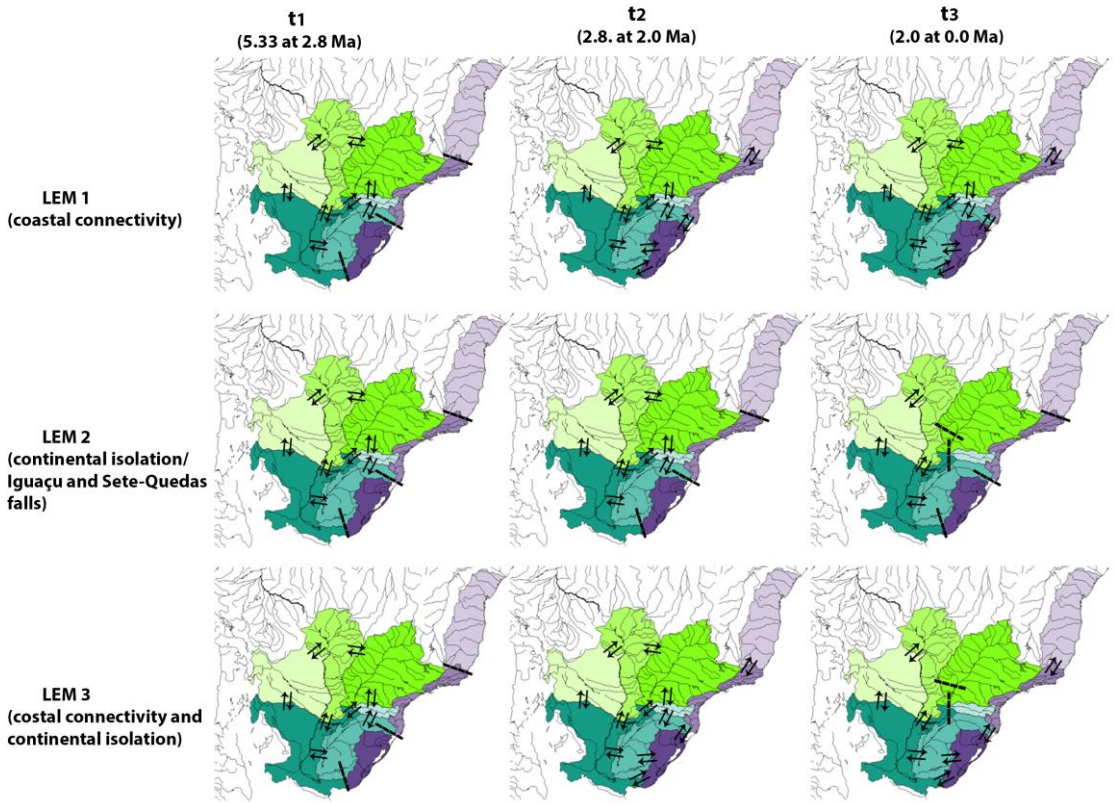


Figure 3. Schematic representation of landscape evolution models (LEMs) within *Oligosarcus* species distribution. **LEM1**: model considers increased connectivity between the coastal areas during the Pleistocene (c. 2.8 Ma). At **t1** sea levels are stable and high and there is no connectivity among coastal drains. However, at **t2-t3**, cyclical sea-level changes facilitates dispersal among coastal basins (including LP and UR areas). **LEM2**: considers the isolation of Iguaçu and upper Paraná after c. 2.0 Ma. At **t1 and t2**, high connectivity and dispersal probability is allowed to all areas within La Plata basin. At **t3**, barriers are formed that isolated Iguaçu and upper Paraná areas. **LEM3**: considers both coastal dispersal events at c. 2.8 Ma and continental isolation of Iguaçu and upper Paraná at c. 2.0 Ma. Black double-headed arrows indicate high dispersal probabilities (rate of 1.0) in the dispersal multiplier matrices. Dashed lines indicate low dispersal probabilities (rates of 0.1 or 0.5).

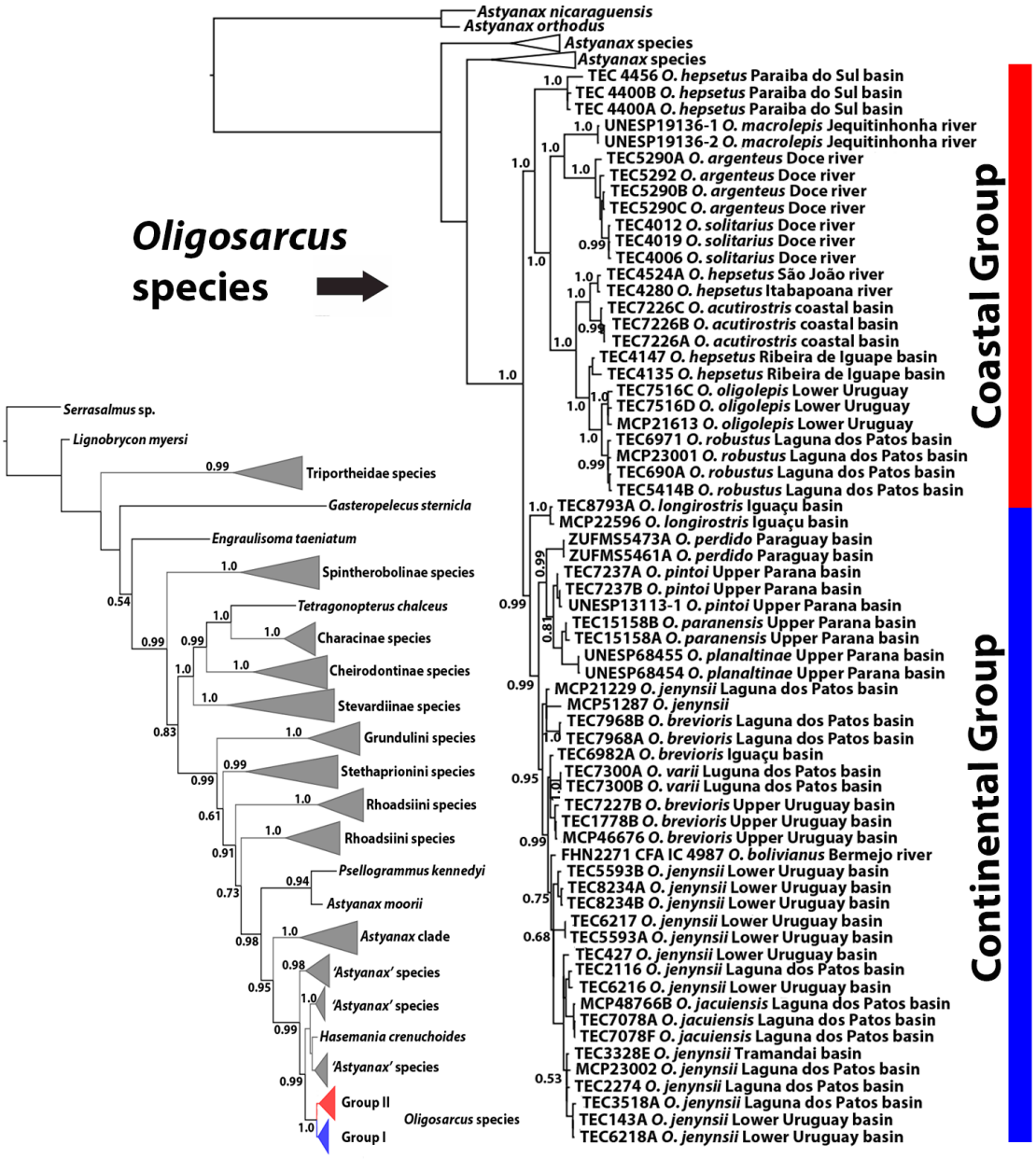
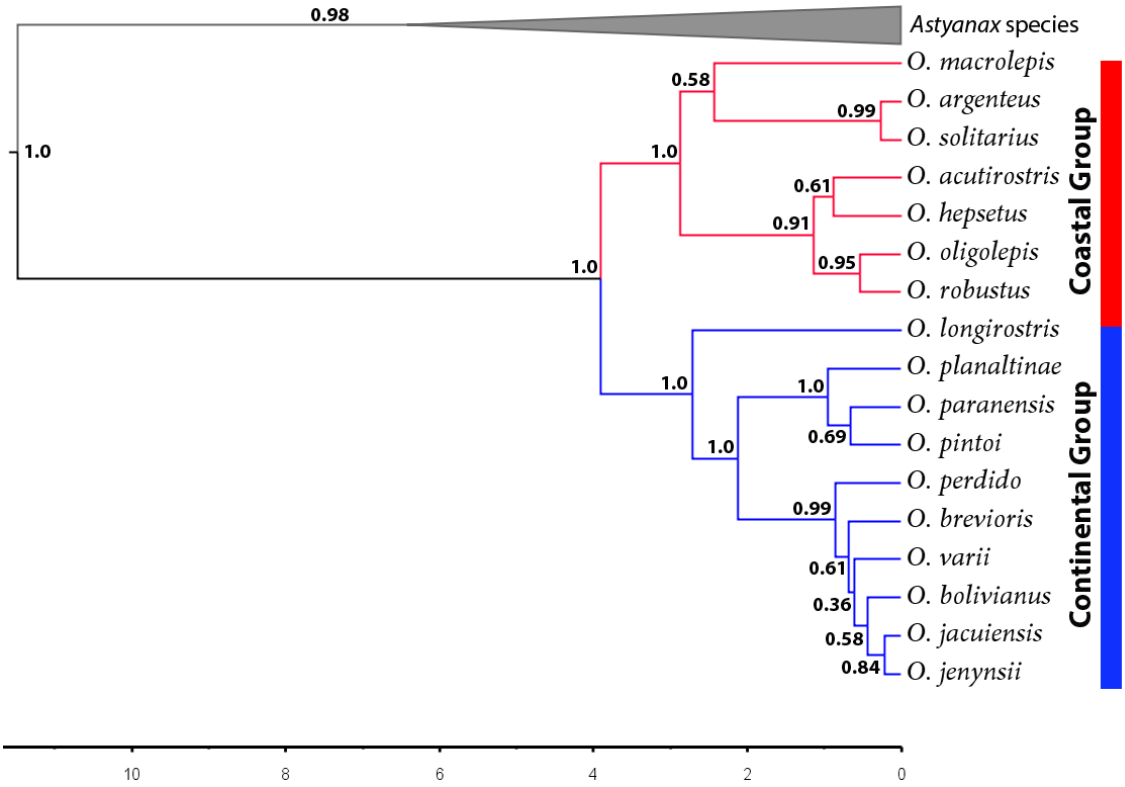
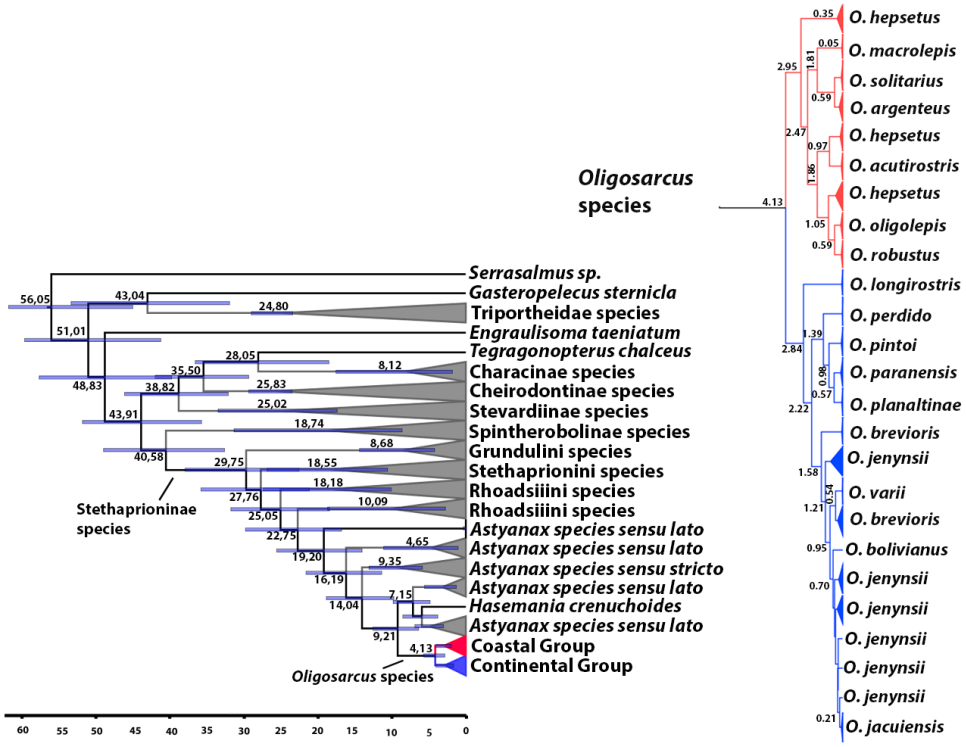


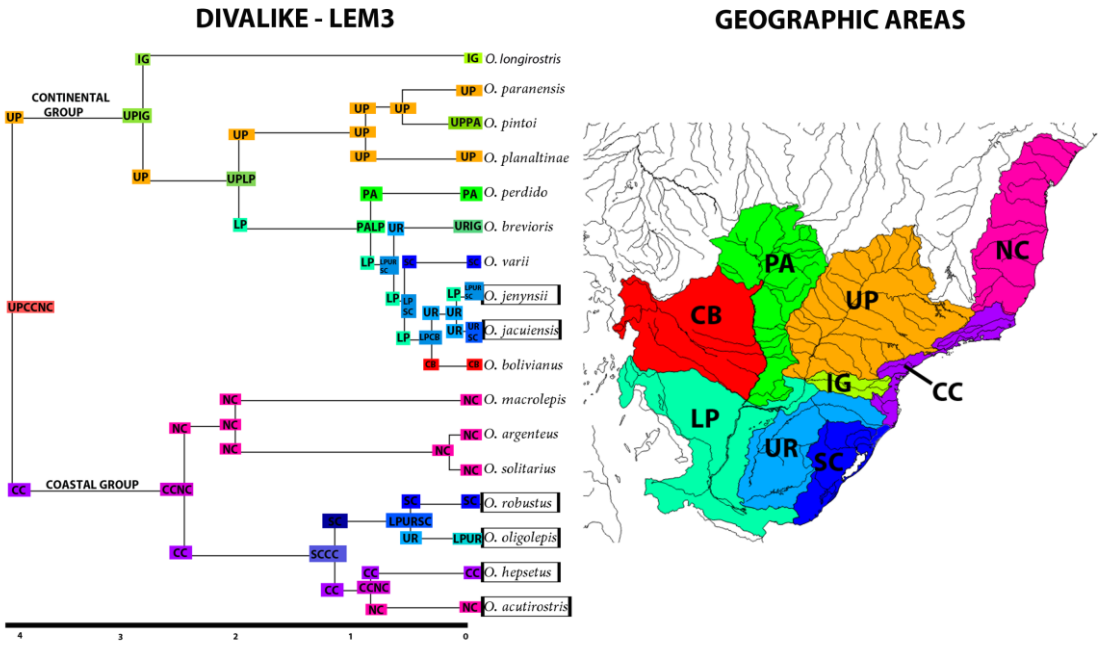
Figure 4. Phylogenetic relationships within *Oligosarcus* species and outgroups based on Bayesian Inference, using concatenated dataset. Posterior probabilities represented by values at the bases of the nodes. Posterior probabilities at species level and clades below 0.5 were not pictured in the phylogeny. A short descriptor of the locality follows species name.



1174 Figure 5. Species Tree of *Oligosarcus* species based on Bayesian Inference. Posterior probabilities
1175 represented by values at the bases of the nodes. Posterior probabilities below 0.5 were not pictured in the
1176 phylogeny. Time bar as Million Years (Ma).



1177 Figure 6. Time-calibrated phylogeny of *Oligosarcus* species and outgroup. Fossils calibrations:
1178 †*Paleotetra* (prior age= 33.9 Ma), †*M. unicus*, and †*L. ligniticus* (both with prior age= 23.03 Ma).
1179 Median age (as Ma) represented by values at the bases of the nodes. Time bar as Million Years (Ma).



1180 Figure 7. Ancestral range estimation for *Oligosarcus* using DIVALIKE model of range
1181 evolution on LEM3. Biogeographic areas: CB=Chaco, PA=Paraguay, UP=Upper Paraná,
1182 LP=Lower Paraná, IG=Iguaçu, UR= Upper and Lower Uruguay ecoregions, NC=North Coastal,
1183 CC= Central Coastal, SC= South Coastal). Black rectangle around terminals indicates the
1184 distribution of species in lowland areas.

1185 Table 1. Comparison of the different models (DEC and DIVALIKE) of ancestral range estimation of
1186 *Oligosarcus* species (with four different scenarios of landscape evolution). M0= null model; LEM1,
1187 LEM2 and LEM3 = landscape evolution models 1, 2 and 3, and different semi-permeability rates (0.1 and
1188 0.5). In bold are the best models, which better fits the geographic evolution of *Oligosarcus*. # number of
1189 estimated parameters; LEM = landscape evolution models; AICc= Akaike information criterion; AICc
1190 weights= AICc weighted; ΔAIC= delta AIC.

Models		**Parameter estimates**				**Likelihood-ratio test**	**Information criteria**		
		Ln *L*	#	*d*	*e*	*P*-value	AICc	AICc weights	ΔAICc
		Without geographical events – M0							
DEC		-60.94	2	0.30	0.34		126.70	0.087	4.7
DIVALIKE		-58.57	2	0.20	1.0e-12	0.00	122.00	0.912	0.0
	LEM	Geographical events and semi-permeable dispersal rate 0.1							
DEC	1	-57.10	2	0.386	0.3025		119.10	0.015	7.70
DIVALIKE	1	-54.56	2	0.2772	0.0295	0.00	114.00	0.201	2.60
DEC	2	−59.22	2	1.4634	0.3342		123.30	0.001	11.90
DIVALIKE	2	−57.99	2	1.1731	0.1634	0.00	120.80	0.006	9.40

DEC	3	-56.31	2	0.5918	0.2974		117.50	0.035	6.10
DIVALIKE	3	-53.29	2	0.3946	1e-12	0.00	111.40	0.739	0.0
	LEM			Geographical events and semi-permeable dispersal rate 0.5					
DEC	1	−59.03	2	0.3365	0.3186		122.90	0.014	7.60
DIVALIKE	1	−56.36	2	0.2297	1e-12	0.00	117.60	0.199	2.30
DEC	2	−59.48	2	0.5122	0.3318		123.80	0.008	8.50
DIVALIKE	2	−56.88	2	0.3271	1e-12	0.00	118.61	0.120	3.31
DEC	3	−58.41	2	0.3947	0.3179		121.70	0.025	6.40
DIVALIKE	3	−55.22	2	0.2719	1e-12	0.00	115.30	0.630	0.0

1191

Supplementary material 1

Table S1. List of taxa, specimens, individual locator, locality, and GenBank accession numbers for *Oligosarcus* and outgroups included in this study. Ecoregions according to FEOW (Abell et al., 2008).

Genera/Species	Catalog number	Reference #/ specimen tag	Country: State: Locality (ecoregion)	COI	RAG 2	ND2	S7	Myh6
Oligosarcus								
O. acutirostris	UFRGS 22533	TEC7226 A	Brazil: Bahia: Santo Antônio River (Northeastern Mata Atlantica)	MN119390	---	MN020376	MN020453	MK991872
	UFRGS 22533	TEC7226 B	Brazil: Bahia: Santo Antônio River (Northeastern Mata Atlantica)	MN119391	MN011637	MN020377	MN020454	---
	UFRGS 22533	TEC7226 C	Brazil: Bahia: Santo Antônio River (Northeastern Mata Atlantica)	MN119392	MN011638	---	MN020455	---
O. argenteus	UFRGS 19745	TEC5290 A	Brazil: Minas Gerais: Doce River (Northeastern Mata Atlantica)	MN119393	MN011639	MN020378	MN020456	---
	UFRGS 19745	TEC5290 B	Brazil: Minas Gerais: Doce River (Northeastern Mata Atlantica)	MN119394	MN011640	---	MN020457	---
	UFRGS 19745	TEC5290 C	Brazil: Minas Gerais: Doce River (Northeastern Mata Atlantica)	MN119395	MN011641	MN020379	MN020458	MK991873
	UFRGS 19747	TEC5292	Brazil: Minas Gerais: Doce River (Northeastern Mata Atlantica)	MN119396	MN011642	---	MN020459	---
O. bolivianus	FHN-2272	CFA-IC-4987	Argentina: Salta: Bermejo River basin (Chaco)	Mirande 2018	---	---	---	---
O. brevioris	UFRGS 14994	TEC1778 B	Brazil: Rio Grande do Sul: Uruguai River (Upper Uruguay)	MN119397	MN011643	MN020380	MN020460	---
	MCP 46676	MCP46676	Brazil: Rio Grande do Sul: Uruguai River (Upper Uruguay)	MN119398	MN011644	MN020381	MN020461	---

	UFRGS 22534	TEC7227 B	Brazil: Rio Grande do Sul: Forquilha river (Upper Uruguay)	MN119399	MN01164 5	MN020382	MN020462	---
	UFRGS 22084	TEC6982 A	Brazil: Paraná: Chopim River (Iguaçu)	MN119400	MN01164 6	MN020383	---	---
	UFRGS 24283	TEC-7968A	Brazil: Rio Grande do Sul: Pinheiro River (Laguna dos Patos)	MN119401	MN01164 7	MN020384	MN020463	---
	UFRGS 24283	TEC-7968B	Brazil: Rio Grande do Sul: Pinheiro River (Laguna dos Patos)	MN119402	MN01164 8	MN020385	MN020464	---
O. hepsetus	UFRGS 18757	TEC4400 A	Brazil: São Paulo: Paraibuna River (Paraíba do Sul)	MN119403	MN01164 9	MN020386	MN020465	---
	UFRGS 18757	TEC4400 B	Brazil: São Paulo: Paraibuna river (Paraiba do Sul)	MN119404	MN01165 0	MN020387	MN020466	---
	UFRGS 16597	TEC2925 A	Brazil: Santa Catarina: Sombrio Lagoon (Southeastern Mata Atlantica)	MN119405	MN01165 1	MN020388	MN119385	---
	UFRGS 16597	TEC2925 B	Brazil: Santa Catarina: Sombrio Lagoon (Southeastern Mata Atlantica)	MN119406	MN01165 2	MN020389	MN119386	---
	UFRGS 18821	TEC4456	Brazil: Rio de Janeiro: Preto River (Paraíba do Sul)	MN119407	MN01165 3	MN020390	MN020467	MK999874
	UFRGS 18901	TEC4524 A	Brazil: Rio de Janeiro: São João River (Fluminense)	MN119408	MN01165 4	MN020391	MN020468	---
	UFRGS 18527	TEC4147	Brazil: São Paulo: Batatal River (Ribeira de Iguape)	MN119409	MN01165 5	MN020392	MN020469	---
	UFRGS 18928	TEC4280	Brazil: Espírito Santo: Muqui do Sul River (Fluminense)	MN119410	MN01165 6	MN020393	MN020470	---
	UFRGS 18522	TEC4135	Brazil: São Paulo: Batatal River (Ribeira de Iguape)	MN119411	MN01165 7	MN020394	MN020471	---
O. jacuiensis	MCP 48766	MCP48766B	Brazil: Rio Grande do Sul: Jacuí River (Laguna dos Patos)	MN119412	MN01165 8	MN020395	---	---
	UFRGS 22247	TEC7078 A	Brazil: Rio Grande do Sul: das Antas River (Laguna dos Patos)	MN119413	MN01165 9	MN020396	MN020472	---

	UFRGS 22247	TEC7078F	Brazil: Rio Grande do Sul: das Antas River (Laguna dos Patos)	MN119414	MN011660	MN020397	MN020473	---
O. jenynsii	UFRGS 10698	TEC143A	Uruguay: Artigas: Arroyo Mandiyú (Lower Uruguay)	MN119415	MN011661	MN020398	MN020474	---
	UFRGS 10989	TEC427	Uruguay: Paysandu: Queguay Grande river (Lower Uruguay)	MN119416	MN011662	MN020399	MN020475	---
	UFRGS 15713	TEC2116	Brazil: Rio Grande do Sul: tributary of Guaíba Lake (Laguna dos Patos)	MN119417	---	MN020400	MN020476	---
	UFRGS 17472	TEC3328E	Brazil: Rio Grande do Sul: Fortaleza Lagoon (Tramandaí-Mampituba)	MN119418	MN011663	MN020401	MN020477	---
	UFRGS 21114	TEC6216	Brazil: Rio Grande do Sul: tributary of Ibicuí River (Lower Uruguay)	---	MN011664	MN020402	MN020478	---
	UFRGS 21117	TEC6217	Brazil: Rio Grande do Sul: Pai Passo Creek (Lower Uruguay)	MN119419	MN011665	---	MN020479	---
	UFRGS 20313	TEC5593A	Brazil: Rio Grande do Sul: Ximbocuzinho River (Lower Uruguay)	MN119420	MN011666	MN020403	MN020480	---
	UFRGS 20313	TEC5593B	Brazil: Rio Grande do Sul: Ximbocuzinho river (Lower Uruguay)	MN119421	---	MN020404	---	---
	UFRGS 21118	TEC6218A	Brazil: Rio Grande do Sul: tributary of Ibicuí River (Lower Uruguay)	MN119422	MN011667	MN020405	MN020481	---
	UFRGS 17839	TEC3518A	Brazil: Rio Grande do Sul: Mirim lagoon (Laguna dos Patos)	MN119423	MN011668	MN020406	MN020482	---
	MCP 21229	MCP21229	Brazil: Rio Grande do Sul: Jacuí River (Laguna dos Patos)	MN119424	MN011669	MN020407	MN020483	---
	MCP 23002	MCP23002	Brazil: Rio Grande do Sul: Jacuí River (Laguna dos Patos)	MN119425	MN011670	MN020408	MN020484	---
	UFRGS 16115	TEC2274	Brazil: Rio Grande do Sul: Mangueira	MN119426	MN011671	MN020409	MN020485	---

			Lagoon (Laguna dos Patos)					
	MCP51287	MCP51287	Brazil: Rio Grande do Sul: Jacutinga River (Upper Uruguay)	---	MN01167 9	MN020415	MN020493	---
O. longirostris	MCP 22596	MCP22596	Brazil: Paraná: Iguaçu River (Iguaçu)	MN119427	MN01167 2	MN020410	MN020486	MK991875
	UFRGS 25342	TEC8793A	Brazil: Paraná: Silva Jardim River (Iguaçu)	MN119428	MN01167 3	---	MN020487	---
O. macrolepis	DZSJRP19136-1	DZSJRP19136-1	Brazil: Minas Gerais: Jequitinhonha River (Northeastern Mata Atlantica)	MN119429	MN01167 4	MN020411	MN020488	---
	DZSJRP19136-2	DZSJRP19136-2	Brazil: Minas Gerais: Jequitinhonha River (Northeastern Mata Atlantica)	MN119430	MN01167 5	MN020412	MN020489	---
O. oligolepis	MCP 21613	MCP21613	Brazil: Rio Grande do Sul: Uruguai River (Lower Uruguay)	MN119431	MN01167 6	---	MN020490	---
	UFRGS 23402	TEC7516C	Brazil: Rio Grande do Sul: Uruguai River (Lower Uruguay)	MN119432	MN01167 7	MN020413	MN020491	---
	UFRGS 23402	TEC7516D	Brazil: Rio Grande do Sul: Uruguai River (Lower Uruguay)	MN119433	MN01167 8	MN020414	MN020492	---
O. paranensis	MZUEL15158	MUZUEL15158A	Brazil: Paraná: Pirapó River (Upper Paraná)	MN119434	MN01168 0	MN020416	MN020494	---
	MZUEL15158	MUZUEL15158B	Brazil: Paraná: Pirapó River (Upper Paraná)	MN119435	MN01168 1	MN020417	MN020495	MK991876
O. perdido	ZUFMS5461	ZUFMS5461A	Brazil: Mato Grosso do Sul: Perdido River (Paraguay)	MN119436	MN01168 2	MN020418	MN020496	---
	ZUFMS5473	ZUFMS5473A	Brazil: Mato Grosso do Sul: Perdido River (Paraguay)	MN119437	MN01168 3	MN020419	MN020497	---
O. pintoi	UFRGS 22535	TEC7237A	Brazil: São Paulo: tributary of Rio Grande River (Upper Paraná)	MN119438	MN01168 4	MN020420	MN020498	MK991877
	UFRGS 22535	TEC7237B	Brazil: São Paulo: tributary of Rio Grande River (Upper Paraná)	MN119439	MN01168 5	MN020421	MN020499	MK991878

	UNESP13113-1	UNESP13113-1	Brazil: São Paulo: tributary of Tietê River (Upper Paraná)	MN119440	MN011686	---	MN020500	---
O. planaltinae	UNESP68454	UNESP68454	Brazil: São Paulo: Paraná River (Upper Paraná)	MN119441	MN011687	MN020422	MN020501	---
	UNESP68455	UNESP68455	Brazil: São Paulo: Paraná River (Upper Paraná)	MN119442	MN011688	MN020423	MN020502	---
O. robustus	UFRGS 22064	TEC6971	Brazil: Rio Grande do Sul: Mirim Lagoon (Laguna dos Patos)	MN119443	MN011689	---	MN020503	---
	UFRGS 10991	TEC690A	Brazil: Rio Grande do Sul: Francisquinho River (Laguna dos Patos)	MN119444	MN011690	MN020424	MN020504	MK991879
	UFRGS 19946	TEC5414B	Brazil: Rio Grande do Sul: Mirim Lagoon (Laguna dos Patos)	MN119445	MN011691	MN020425	MN020505	MK991880
	MCP23001	MCP23001	Brazil: Rio Grande do Sul: Jacuí River (Laguna dos Patos)	MN119446	MN011692	---	MN020506	---
O. solitarius	UFRGS 19056	TEC4019	Brazil: Minas Gerais: Doce River (Northeastern Mata Atlantica)	MN119447	MN011693	MN020426	MN020507	---
	UFRGS 19056	TEC4006	Brazil: Minas Gerais: Doce River (Northeastern Mata Atlantica)	MN119448	MN011694	MN020427	MN020508	---
	UFRGS 19056	TEC4012	Brazil: Minas Gerais: Doce River (Northeastern Mata Atlantica)	MN119449	MN011695	---	MN020509	---
O. varii	UFRGS 22701	TEC7300A	Brazil: Rio Grande do Sul: São Marcos River (Laguna dos Patos)	MN119450	MN011696	MN020428	MN020510	---
	UFRGS 22701	TEC7300B	Brazil: Rio Grande do Sul: São Marcos River (Laguna dos Patos)	MN119451	MN011697	MN020429	MN020511	---
Oligosarcus sp.	UFRGS 24673	TEC8234A	Brazil: Rio Grande do Sul: Turvo River (Lower Uruguay)	MN119452	MN011698	MN020430	MN020512	---
	UFRGS 24673	TEC8234B	Brazil: Rio Grande do Sul: Turvo river (Lower Uruguay)	MN119453	MN011699	MN020431	MN020513	---

Outgroup Characidae								
Stethaprioninae								
Astyanax bagual	UFRGS17834	TEC3513	Brazil: Rio Grande do Sul: Laguna dos Patos	MN119454	---	MN020433	---	---
Astyanax brachpterigyum	UNFRGS21849	TEC6844	Brazil: Rio Grande do Sul: Pelotas River (Laguna dos Patos)	MN119455		MN020434		MK998882
Astyanax cremnobates	UFRGS 18430	TEC 3823B	Brazil: Rio Grande do Sul: Laguna dos Patos	MN119456	---	---	---	---
	UFRGS 18430	TEC3823C	Brazil: Rio Grande do Sul: Laguna dos Patos	MN119457	---	---	---	---
Astyanax dissensus	UFRGS 16521	TEC3225B	Brazil: Rio Grande do Sul: Laguna dos Patos	MN119458	---	---	---	---
	UFRGS 16521	TEC3325C	Brazil: Rio Grande do Sul: Laguna dos Patos	MN119459	---	---	---	MK998883
Astyanax douradilho	UFRGS18444	TEC3837A	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	MN119460	---	MN020435	---	---
	UFRGS18444	TEC3837B	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	MN119461	---	MN020436	---	---
Astyanax eigenmanniorum	UFRGS19221	TEC4916A	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	MN119462	MN01170	MN020437	MN020514	---
	UFRGS19221	TEC4916B	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	MN119463	MN01171	MN020438	MN020515	---
Astyanax fasciatus	UFRGS19135	TEC4853A	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	KY327450	---	MN020439	---	MK998884
	UFRGS19135	TEC4853B	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	KY327451	MN01172	MN020440	MN020516	MK998885
Astyanax henseli	UFRGS19598	TEC5189A	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	MN119464	---	MN020441	---	MK998886
	UFRGS19598	TEC5189B	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	MN119465	---	---	---	MK998887
Astyanax lacustris	UFRGS15350	TEC1911	Brazil: São Paulo: Upper Paraná	---	---	MN020432	---	MK998881
	UFRGS19151	TEC4869A	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	MN119466	MN01173	---	---	---
	UFRGS19151	TEC4869B	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	MH029369	MN01174	---	---	---

	UFRGS19055	TEC4030	Brazil: Minas Gerais: Nordeste da Mata Atlântica	KY327441	---	MN020442	---	MK991888
	UFRGS19055	TEC4783	Brazil: Espírito Santo: Engano River	MN119467	---	---	---	---
Astyanax sp.	UFRGS19746	TEC5291E	Brazil: Tripuí River, Doce River basin (Northeast Mata Atlantica)	MN119468	---	MN020443	---	MK991889
Astyanax sp.	UFRGS19746	TEC5291A	Brazil: Tripuí River, Doce River basin (Northeast Mata Atlantica)	MN119469	---	MN020444	---	MK991890
Astyanax paranae	UFRGS15071	TEC1855	Brazil: São Paulo: Alto Paraná	MN119470	---	MN020445	MN020517	MK991891
Astyanax rivularis	UFRGS11375	TEC1213	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	MN119471	---	MN020446	---	MK991892
Astyanax scabripinnis	MZUFV 4456	CT2772	Brazil: Doce River basin (Northeast Mata Atlantica)	KY327444	---	MN020447	---	MK991893
	MZUFV 4456	CT2773	Brazil: Doce River basin (Northeast Mata Atlantica)	KY327445	MN011705	MN020448	MN020518	MK991894
Astyanax xiru	UFRGS18438	TEC3831	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	MN119472	MN011706	MN020449	MN020519	MK991895
	UFRGS19607	TEC5197A	Brazil: Rio Grande do Sul: Tramandaí-Mampituba	MN119473	---	---	---	---
Astyanax aeneus	LBP8938	42019	México: Quintana Roo: Chichancanab lagoon	---	HQ289511	---	---	HQ289126
Astyanax jordani	LBP4511	24599	Brazil: Aquarium	---	HQ289423	---	---	HQ289036
Astyanax mexicanus	UFRGS 22645	TEC 7255B	México: Aquarium	MN119474	---	---	MN020521	MK991899
Astyanax nasutus	A1480			FJ439388	---	---	---	---
Astyanax nicaraguensis	A898NI			FJ439379	---	---	---	---
Astyanax baileyi	LBP8940	42025	Guatemala: Alta Verapaz: Chisec: Chajmaic	---	HQ289513	---	---	HQ289128
Astyanax caballeroi	LBP8939	42022	México: Vera Cruz: Catemaco	---	HQ289512	---	---	HQ289127
Astyanax moorii	LBP5783	28195	Brazil: Minas Gerais: Muzambinho River	---	HQ289447	---	---	HQ289061

Astyanax orthodus	JJ28			FJ439407	---	---	---	---
Astyanax intermedius	MZUFV 4458	CT2801	Brazil: Doce River basin	KY327432	---	MN020452	---	MK991898
Deuterodon iguape	UFRGS 20032	TEC4130	Brazil: São Paulo: Ribeira de Iguape River	KY327421	MN011707	MN020450	MN020520	MK991896
	UFRGS 18525	TEC4138	Brazil: São Paulo: Ribeira de Iguape River	KY327420	---	MN020451	---	MK991897
Hasemania crenuchoides	LBPV-33197			JN988883	---	---	---	---
Hollandichthys multifasciatus	UFRGS11793	TEC842E	Brazil: Rio Grande do Sul: Maquiné River (Tramandaí-Mampituba)	HM562852	---	HM562883	---	KF210427
Hyphessobrycon eques	RJ DNA-14			FJ749057	FJ749085	---	---	---
Hyphessobrycon megalopterus	RJ DNA-16			FJ749058	FJ749100	---	---	---
Myxiops aphos	UFBA 07798	A	Brazil: Paraguaçu drainage	KY327452	---	---	---	MN119387
Myxiops aphos	UFBA 07798	B	Brazil: Paraguaçu drainage	KY327453	---	---	---	MN119388
Nematobrycon palmeri	RJ DNA-22			FJ749061	FJ749103	---	---	HQ289075
Paracheirodon axelrodi	RJ DNA-21			FJ749060	FJ749102	---	---	---
Paracheirodon innesi	KW11T043			KU568960	---	---	---	---
Probolodus heterostomus	UFRGS18758	TEC4184	Brazil: Paraíbuna and Paraíba do Sul River basin	KY327456	---	MN119384	---	MN119389
Psellogrammus kennedyi	LBPV-31814			JN989171	---	---	---	---
Rachoviscus crassiceps	UFRGS9356	TEC102	Brazil: Santa Catarina: Southeastern Mata Atlantica	HM562857	FJ749107	---	---	---
Rachoviscus graciliceps	RJ Rgr1			FJ749079	FJ749106	HM562888	---	---
Rhoadsia altipinna	MUGT:P-1572-703			KY440350	---	---	---	---
Spintherobolinae								
Amazonspinther dalmata	LBP9309	46005		---	KC196385	---	---	---

Spintherobolus leptoura	LBP7544	36098	Brazil: São Paulo: Mumuna River tributary	MG967588	HQ289486	---	---	HQ289101
Spintherobolus ankoseion	LBP4725	24957	Brazil: Santa Catarina: São Francisco do Sul: Acaraí lagoon	---	HQ289427	---	---	HQ289040
Spintherobolus broccae	LBP3916	22558	Brazil: São Paulo: Vermelho River tributary	---	HQ289391	---	---	HQ289004
Stevardiinae								
Bryconamericus iheringii	UFRGS10002	TEC 697	Brazil: Rio Grande do Sul: Laguna dos Patos	FJ749041	FJ749114	---	---	KF210313
Bryconamericus patriciae	UFRGS8205	TEC 716	Brazil: Santa Catarina: Pelotas River (Upper Uruguay)	FJ749042	FJ749111	---	---	KF210321
Diapoma uruguayensis	UFRGS10000	TEC46	Uruguay: Rivera: Tacuarembó River	FJ749049	FJ749108	KP406707	---	---
Diapoma dicropotamicus	UFRGS12727	TEC1465A	Brazil: Rio Grande do Sul: Prata River (Laguna dos Patos)	KP399736	---	KP406705	---	KF210398
Diapoma alegretensis	UFRGS10008	TEC714	Brazil: Uruguai basin	FJ749047	FJ749117	KP406706	---	KF210395
Diapoma guarani	UFRGS12647	TEC1379A	Brazil: Rio Grande do Sul: Lower Uruguay	KF210246	KF211231	---	---	---
Eretmobrycon emperador	STRI861		Colombia: San Juan	KF210059	---	KF211030	---	KF210307
Eretmobrycon bayano	STRI7334		Panamá: Bayano	---	KF211126	---	---	---
Eretmobrycon dahli	STRI9302		Colombia: Patia: Guachicono River	KF210052	KF211022	---	---	KF210301
Eretmobrycon peruanus	STRI15003		Perú: Cañete	KF210071	KF211050	---	---	KF210323
Odontostoechus sp.	MCP23595	MCP23595	Brazil: Santa Catarina: Tramandaí-Mampituba	?	?	---	---	KF210543
Markiana nigripinnis	LBP663	8038	Brazil: Mato Grosso: Pirai River tributary	---	HQ289524	---	---	HQ289140
Markiana cf nigripinnis	UFRGS11794	TEC1160A	Brazil: Mato Grosso: Paraguay system	KF210234	---	---	---	KF210528
Markiana cf nigripinnis	UFRGS11794	TEC1160B	Brazil: Mato Grosso: Paraguay system	KF210235	---	---	---	KF210529
Piabarchus stramineus	UFRGS12898		Brazil: Minas Gerais: Doce stream (São Francisco River)	KF210084	KF211070.1	---	---	KF210341
Cheirodontinae								

Cheirodon ibicuhiensis	UFRGS12508	TEC1326A	Brazil: Rio Grande do Sul: Pelotas stream (Laguna dos Patos)	KF210149	KF211131	---	---	KF210424
Heterocheirodon yatai	LBP4872	24954	Urugay: Durazno: Yi River	---	HQ289426	---	---	HQ289039
Prodontocharax sp	MNHG2725025	1525	Perú: Huallaga	KF210156	AY804109	---	---	---
Protocheirodon pi	LBP49257			---	JQ820026	---	---	JQ820056
Pseudocheirodon arnoldi	STRI-00971			MG937160	HQ289522	---	---	HQ289138
Characinae								
Charax stenopterus	UFRGS12611		Brazil: Rio Grande do Sul: Tramandaí-Mampituba	KF210147	KF211129	---	---	KF210422
Roeboides sp	AMNH 233430			---	AY804056	---	---	---
Tetragonopterinae								
Tetragonopterus chalceus	MCP30336	MCP30336	Brazil: Mato Grosso: Teles Pires River	FJ749080	FJ749091	---	---	HQ289113.1
Triportheidae								
Agoniates anchovia	LBP6740	33471	Brazil: Amazonas/Manaus: Catalão Lagoon	---	HQ289472	---	---	---
Agoniates halecinus	LBP5503	26594	Brazil: Amapá: Laranjal do Jari	---	HQ289437	---	---	HQ289051
Clupeacharax anchoveoides	LBP5046	26012	Brazil: Mato Grosso: Cáceres	---	HQ289433	---	---	HQ289046
Lignobrycon myersi	LBP8094	37519	Brazil: Bahia: Braço River	---	HQ289495	---	---	HQ289110
Gasteropelecidae								
Engraulisoma taeniatum	LBP4038	22897	Brazil: Acre: Moa River	---	HQ289396	---	---	---
Gasteropelecus sternicla	LBP4070	22975	Brazil: Acre: Japiim River	---	HQ289400	---	---	HQ289014
Serrasalmidae								
Serrasalmus sp.	UFRGS 12672	TEC 1410	Brazil: Mato Grosso: Paraguay system	KF210158	KF211147.1	---	---	---

Table S2. Primers, references and PCR conditions used in this study.

Gene	Primers name	Primer sequences (liste from 5' to 3')	Reference	Denaturation	Cycles	Extension
COI	*FishF2_t1	CGACTAATCATAAAGATATCGGCAC	Ivanova *et al.* 2007	94°C/3’	35x 94°C/30”, 52°C/40”, 72°C/1’	72°C/10'
	*VF2_t1	CAACCAACCACAAAGACATTGGCAC				
	**FishR2_t1	ACTTCAGGGTGACCGAAGAATCAGAA	Ivanova *et al.* 2007	94°C/3’	35x 94°C/30”, 52°C/40”, 72°C/1’	72°C/10'
	**FR1d_t1	ACCTCAGGGTGTCCGAARAAYCARAA				
	COI L6252	AAGGCGGGGAAAGCCCCGGCAG	Melo *et al*., 2011	95°C/4’	35x 95°C/30”, 50°C/45”, 72°C/45”	72°C/10'
	COI H7271	TCCTATGTAGCCGAATGGTTCTTTT				
ND2	ND2 – F	AAYTTGTWAAACTCACGATGCTCTC	Present study	94°C/4’	35x 95°C/30”, 60°C/45”, 72°C/1’30”	72°C/10'
	ND2 - R	ATAATAAGGGGTGCTAKGGGTAAAA				
RAG2	[1]RAG2 164F	AGCTCAAGCTGCGYGCCAT	Oliveira *et al*., 2011	94°C/5’	35x 94°C/1’, 50°C/1’, 72°C/1'30"	72°C/5'
	[2]RAG2-R6	TGRTCCARGCAGAAGTACTTG				
	[1]RAG2 176R	GYGCCATCTCATTCTCCAACA	Oliveira *et al*., 2011	94°C/5’	35x 94°C/1’, 50°C/1’, 72°C/1'30"	72°C/5'
	[2]RAG2 Rag2Ri	AGAACAAAAGATCATTGCTGGTCGGG				
S7	S7RPEX1-F	TGGCCTCTTCCTTGGCCGTC	Chow & Hazama (1998)	95°C/1’	30x 95°C/30”, 56°C/1’, 72°C/2'	72°C/10'
	S7RPEX2-R	AACTCGTCTGGCTTTTCGCC				
Myh6 1stPCR	F459	CATMTTYTCCATCTCAGATAATGC	Li *et al*. (2007)	94°C/3’	35x 94°C/30”, 53°C/45”, 72°C/1'30"	72°C/10'
	R1325	ATTCTCACCACCATCCAGTTGAA				
Myh6 2ndPCR	F507	GGAGAATCARTCKGTGCTCATCA	Li *et al*. (2007)	94°C/3’	35x 94°C/30”, 62°C/45”, 72°C/1'30"	72°C/10'
	R1322	CTCACCACCATCCAGTTGAACAT				

* Primers that compound the cocktail FishF1t1; ** Primers that compound the cocktail FishR1t1; [1] Primers that compound the cocktail RAG2-F; [2] Primers that compound the cocktail RAG2-R.

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Table S3. Genes partitioned by codon position and the best nucleotide substitution model and partition scheme obtained by PartitionFinder using BIC criteria for each of the analyses.

Gene/partition	**Position**	*** Linked partitions**	**Best model for concatenated (MrBayes)**
COI 1st position	1–714/3	1	SYM+I+G
COI 2nd position	2–714/3	2	F81
COI 3rd position	2–714/3	3	GTR+G
ND2 1st position	715–1720/3	4	GTR+I+G
ND2 2nd position	716–1720/3	5	GTR+G
ND2 3rd position	717–1720/3	3	GTR+G
Rag2 1st position	1721–2803/3	6	K80+I+G
Rag2 2nd position	1722–2803/3	6	K80+I+G
Rag2 3rd position	1723–2803/3	7	SYM+G
MYH6 1st position	2804-3578\3	8	K80+I+G
MYH6 2nd position	2805-3578\3	8	K80+I+G
MYH6 3rd position	2806-3578\3	7	SYM+G
S7	3579-4321	9	HKY+G
			Best model for SpeciesTree (StarBeast)
COI 1st position	1–714/3	1	TrN+I+G
COI 2nd position	2–714/3	2	K80+I+G
COI 3rd position	2–714/3	3	TrN+G
ND2 1st position	715–1720/3	4	K80+G
ND2 2nd position	716–1720/3	2	TrN+G
ND2 3rd position	717–1720/3	3	TrN+G
Rag2 1st position	1721–2803/3	1	TrN+I+G
Rag2 2nd position	1722–2803/3	1	K80+I+G
Rag2 3rd position	1723–2803/3	4	K80+G
MYH6 1st position	2804-3578\3	1	K80+I+G
MYH6 2nd position	2805-3578\3	1	K80+I+G
MYH6 3rd position	2806-3578\3	4	K80+I
S7	3579-4321	5	HKY+I

			Best model for dating analysis (Beast)
COI 1st position	1–714/3	1	TrNef+I+G
COI 2nd position	2–714/3	2	HKY
COI 3rd position	2–714/3	3	TrN+G
ND2 1st position	715–1720/3	4	GTR+I+G
ND2 2nd position	716–1720/3	5	GTR+G
ND2 3rd position	717–1720/3	3	TrN+G
Rag2 1st position	1721–2803/3	6	K80+G
Rag2 2nd position	1722–2803/3	7	HKY+I+G
Rag2 3rd position	1723–2803/3	8	SYM+G
MYH6 1st position	2804-3578\3	7	HKY+I+G
MYH6 2nd position	2805-3578\3	7	HKY+I+G
MYH6 3rd position	2806-3578\3	8	SYM+G
S7	3579-4321	9	HKY+G
			Best model for dating analysis (RAxML)
COI 1st position	1–714/3	1	GTR+G
COI 2nd position	2–714/3	2	GTR+G
COI 3rd position	2–714/3	3	GTR+G
ND2 1st position	715–1720/3	4	GTR+G
ND2 2nd position	716–1720/3	5	GTR+G
ND2 3rd position	717–1720/3	3	GTR+G
Rag2 1st position	1721–2803/3	6	GTR+G
Rag2 2nd position	1722–2803/3	2	GTR+G
Rag2 3rd position	1723–2803/3	5	GTR+G
MYH6 1st position	2804-3578\3	6	GTR+G
MYH6 2nd position	2805-3578\3	2	GTR+G
MYH6 3rd position	2806-3578\3	5	GTR+G
S7	3579-4321	7	GTR+G

* linked partitions were indicated by the same number

Table S4 Matrix of presence or absence of species of *Oligosarcus* in geographical units. 0= species absent in the area; 1= species present in the area. CB= Chaco; UP = Upper Paraná; IG = Iguaçu; PA = Paraguay; LP = Lower Paraná; UR = Upper and Lower Uruguay; SC = South Coastal; CC = Central Coastal; NC = North Coastal.

	CB	UP	IG	PA	LP	UR	SC	CC	NC
O. acutirostris	0	0	0	0	0	0	0	0	1
O. argenteus	0	0	0	0	0	0	0	0	1
O. bolivianus	1	0	0	0	0	0	0	0	0
O. brevioris	0	0	1	0	0	1	0	0	0
O. hepsetus	0	0	0	0	0	0	0	1	0
O. jacuiensis	0	0	0	0	0	1	1	0	0
O. jenynsii	0	0	0	0	1	1	1	0	0
O. longirostris	0	0	1	0	0	0	0	0	0
O. macrolepis	0	0	0	0	0	0	0	0	1
O. oligolepis	0	0	0	0	1	1	0	0	0
O. paranensis	0	1	0	0	0	0	0	0	0
O. perdido	0	0	0	1	0	0	0	0	0
O. pintoi	0	1	0	1	0	0	0	0	0
O. planaltinae	0	1	0	0	0	0	0	0	0
O. robustus	0	0	0	0	0	0	1	0	0
O. solitarius	0	0	0	0	0	0	0	0	1
O. varii	0	0	0	0	0	0	1	0	0

Table S5. Adjacency area matrix. 0 = area not adjacent, 1 = area adjacent. Areas: CB= Chaco; UP = Upper Paraná; IG = Iguaçu; PA = Paraguay; LP = Lower Paraná; UR = Upper and Lower Uruguay; SC = South Coastal; CC = Central Coastal; NC = North Coastal.

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	0	0	1	1	0	0	0	0
UP	0	1	1	1	1	0	0	1	1
IG	0	1	1	0	1	1	0	1	0
PA	1	1	0	1	1	0	0	0	0
LP	1	1	1	1	1	1	1	0	0
UR	0	0	1	0	1	1	1	1	0
SC	0	0	0	0	1	1	1	1	0
CC	0	1	1	0	0	1	1	1	1
NC	0	1	0	0	0	0	0	1	1

Table S6. Dispersal multiplier matrices to LEM with quasi-impermeable barriers = 0.1. From top to bottom 0-2.0, 2.0-2.8 and 2.8-5.3 Ma time slices.

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.1	0.1	0.1
UP	1	1	1	1	1	1	0.1	0.1	0.1
IG	1	1	1	1	1	1	0.1	0.1	0.1
PA	1	1	1	1	1	1	0.1	0.1	0.1
LP	1	1	1	1	1	1	1	1	1
UR	1	1	1	1	1	1	1	1	1
SC	0.1	0.1	0.1	0.1	1	1	1	1	1
CC	0.1	0.1	0.1	0.1	1	1	1	1	1
NC	0.1	0.1	0.1	0.1	1	1	1	1	1

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.1	0.1	0.1
UP	1	1	1	1	1	1	0.1	0.1	0.1
IG	1	1	1	1	1	1	0.1	0.1	0.1
PA	1	1	1	1	1	1	0.1	0.1	0.1
LP	1	1	1	1	1	1	1	1	1
UR	1	1	1	1	1	1	1	1	1
SC	0.1	0.1	0.1	0.1	1	1	1	1	1
CC	0.1	0.1	0.1	0.1	1	1	1	1	1
NC	0.1	0.1	0.1	0.1	1	1	1	1	1

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.1	0.1	0.1
UP	1	1	1	1	1	1	0.1	0.1	0.1
IG	1	1	1	1	1	1	0.1	0.1	0.1
PA	1	1	1	1	1	1	0.1	0.1	0.1
LP	1	1	1	1	1	1	0.1	0.1	0.1
UR	1	1	1	1	1	1	0.1	0.1	0.1
SC	0.1	0.1	0.1	0.1	0.1	0.1	1	0.1	0.1

CC	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1	0.1
NC	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1

END

Table S7. Dispersal multiplier matrices to LEM 2 with quasi-impermeable barriers = 0.1. From top to bottom 0-2.0, 2.0-2.8 and 2.8-5.3 Ma time slices.

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	0.1	0.1	1	1	1	0.1	0.1	0.1
UP	0.1	1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
IG	0.1	0.1	1	0.1	0.1	0.1	0.1	0.1	0.1
PA	1	0.1	0.1	1	1	1	0.1	0.1	0.1
LP	1	0.1	0.1	1	1	1	0.1	0.1	0.1
UR	1	0.1	0.1	1	1	1	0.1	0.1	0.1
SC	0.1	0.1	0.1	0.1	0.1	0.1	1	0.1	0.1
CC	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1	0.1
NC	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.1	0.1	0.1
UP	1	1	1	1	1	1	0.1	0.1	0.1
IG	1	1	1	1	1	1	0.1	0.1	0.1
PA	1	1	1	1	1	1	0.1	0.1	0.1
LP	1	1	1	1	1	1	0.1	0.1	0.1
UR	1	1	1	1	1	1	0.1	0.1	0.1
SC	0.1	0.1	0.1	0.1	0.1	0.1	1	0.1	0.1
CC	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1	0.1
NC	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.1	0.1	0.1
UP	1	1	1	1	1	1	0.1	0.1	0.1
IG	1	1	1	1	1	1	0.1	0.1	0.1
PA	1	1	1	1	1	1	0.1	0.1	0.1
LP	1	1	1	1	1	1	0.1	0.1	0.1
UR	1	1	1	1	1	1	0.1	0.1	0.1
SC	0.1	0.1	0.1	0.1	0.1	0.1	1	0.1	0.1
CC	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1	0.1
NC	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1

END

Table S8. Dispersal multiplier matrices to LEM 3 with quasi-impermeable barriers = 0.1. From top to bottom 0-2.0, 2.0-2.8 and 2.8-5.3 Ma time slices.

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	0.1	0.1	1	1	1	0.1	0.1	0.1
UP	0.1	1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
IG	0.1	0.1	1	0.1	0.1	0.1	0.1	0.1	0.1
PA	1	0.1	0.1	1	1	1	0.1	0.1	0.1
LP	1	0.1	0.1	1	1	1	1	1	1
UR	1	0.1	0.1	1	1	1	1	1	1
SC	0.1	0.1	0.1	0.1	1	1	1	1	1
CC	0.1	0.1	0.1	0.1	1	1	1	1	1

NC	0.1	0.1	0.1	0.1	1	1	1	1	1

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.1	0.1	0.1
UP	1	1	1	1	1	1	0.1	0.1	0.1
IG	1	1	1	1	1	1	0.1	0.1	0.1
PA	1	1	1	1	1	1	0.1	0.1	0.1
LP	1	1	1	1	1	1	1	1	1
UR	1	1	1	1	1	1	1	1	1
SC	0.1	0.1	0.1	0.1	1	1	1	1	1
CC	0.1	0.1	0.1	0.1	1	1	1	1	1
NC	0.1	0.1	0.1	0.1	1	1	1	1	1

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.1	0.1	0.1
UP	1	1	1	1	1	1	0.1	0.1	0.1
IG	1	1	1	1	1	1	0.1	0.1	0.1
PA	1	1	1	1	1	1	0.1	0.1	0.1
LP	1	1	1	1	1	1	0.1	0.1	0.1
UR	1	1	1	1	1	1	0.1	0.1	0.1
SC	0.1	0.1	0.1	0.1	0.1	0.1	1	0.1	0.1
CC	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1	0.1
NC	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1

END

Table S9. Dispersal multiplier matrices to LEM 1 with semi-permeable barriers = 0.5. From top to bottom 0-2.0, 2.0-2.8 and 2.8-5.3 Ma time slices.

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.5	0.5	0.5
UP	1	1	1	1	1	1	0.5	0.5	0.5
IG	1	1	1	1	1	1	0.5	0.5	0.5
PA	1	1	1	1	1	1	0.5	0.5	0.5
LP	1	1	1	1	1	1	1	1	1
UR	1	1	1	1	1	1	1	1	1
SC	0.5	0.5	0.5	0.5	1	1	1	1	1
CC	0.5	0.5	0.5	0.5	1	1	1	1	1
NC	0.5	0.5	0.5	0.5	1	1	1	1	1

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.5	0.5	0.5
UP	1	1	1	1	1	1	0.5	0.5	0.5
IG	1	1	1	1	1	1	0.5	0.5	0.5
PA	1	1	1	1	1	1	0.5	0.5	0.5
LP	1	1	1	1	1	1	1	1	1
UR	1	1	1	1	1	1	1	1	1
SC	0.5	0.5	0.5	0.5	1	1	1	1	1
CC	0.5	0.5	0.5	0.5	1	1	1	1	1
NC	0.5	0.5	0.5	0.5	1	1	1	1	1

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.5	0.5	0.5

UP	1	1	1	1	1	1	0.5	0.5	0.5
IG	1	1	1	1	1	1	0.5	0.5	0.5
PA	1	1	1	1	1	1	0.5	0.5	0.5
LP	1	1	1	1	1	1	0.5	0.5	0.5
UR	1	1	1	1	1	1	0.5	0.5	0.5
SC	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5	0.5
CC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5
NC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1

END

Table S10. Dispersal multiplier matrices to LEM 2 with semi-permeable barriers = 0.5. From top to bottom 0-2.0, 2.0-2.8 and 2.8-5.3 Ma time slices.

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	0.5	0.5	1	1	1	0.5	0.5	0.5
UP	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5
IG	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5
PA	1	0.5	0.5	1	1	1	0.5	0.5	0.5
LP	1	0.5	0.5	1	1	1	0.5	0.5	0.5
UR	1	0.5	0.5	1	1	1	0.5	0.5	0.5
SC	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5	0.5
CC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5
NC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.5	0.5	0.5
UP	1	1	1	1	1	1	0.5	0.5	0.5
IG	1	1	1	1	1	1	0.5	0.5	0.5
PA	1	1	1	1	1	1	0.5	0.5	0.5
LP	1	1	1	1	1	1	0.5	0.5	0.5
UR	1	1	1	1	1	1	0.5	0.5	0.5
SC	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5	0.5
CC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5
NC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.5	0.5	0.5
UP	1	1	1	1	1	1	0.5	0.5	0.5
IG	1	1	1	1	1	1	0.5	0.5	0.5
PA	1	1	1	1	1	1	0.5	0.5	0.5
LP	1	1	1	1	1	1	0.5	0.5	0.5
UR	1	1	1	1	1	1	0.5	0.5	0.5
SC	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5	0.5
CC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5
NC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1

END

Table S11. Dispersal multiplier matrices to LEM 3 with semi-permeable barriers = 0.5. From top to bottom 0-2.0, 2.0-2.8 and 2.8-5.3 Ma time slices.

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	0.5	0.5	1	1	1	0.5	0.5	0.5

UP	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5
IG	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5
PA	1	0.5	0.5	1	1	1	0.5	0.5	0.5
LP	1	0.5	0.5	1	1	1	1	1	1
UR	1	0.5	0.5	1	1	1	1	1	1
SC	0.5	0.5	0.5	0.5	1	1	1	1	1
CC	0.5	0.5	0.5	0.5	1	1	1	1	1
NC	0.5	0.5	0.5	0.5	1	1	1	1	1

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.5	0.5	0.5
UP	1	1	1	1	1	1	0.5	0.5	0.5
IG	1	1	1	1	1	1	0.5	0.5	0.5
PA	1	1	1	1	1	1	0.5	0.5	0.5
LP	1	1	1	1	1	1	1	1	1
UR	1	1	1	1	1	1	1	1	1
SC	0.5	0.5	0.5	0.5	1	1	1	1	1
CC	0.5	0.5	0.5	0.5	1	1	1	1	1
NC	0.5	0.5	0.5	0.5	1	1	1	1	1

	CB	UP	IG	PA	LP	UR	SC	CC	NC
CB	1	1	1	1	1	1	0.5	0.5	0.5
UP	1	1	1	1	1	1	0.5	0.5	0.5
IG	1	1	1	1	1	1	0.5	0.5	0.5
PA	1	1	1	1	1	1	0.5	0.5	0.5
LP	1	1	1	1	1	1	0.5	0.5	0.5
UR	1	1	1	1	1	1	0.5	0.5	0.5
SC	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5	0.5
CC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5
NC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1

END

Table S12. Nucleotide composition for the molecular dataset used to infer the phylogeny of *Oligosarcus*.

	Gene				
	COI	*ND2*	*RAG2*	*MYH6*	*S7*
Number of sequences	130	81	152	66	69
Number of base pairs after alignment	714	1006	1083	782	743
Number of variable sites	292	683	460	233	129
Number of information sites for parsimony	264	615	323	173	57
% of informative characters for parsimony	37	61	29	22	7
ΠA	23.5	32.3	24.1	30.7	27.1
ΠC	26.5	28.0	26.0	21.4	13.2
ΠG	18.7	13.1	27.4	23.2	28.7
ΠT	31.3	26.6	22.4	24.7	30.9
Mean genetic distance (p-distance)	0.162	0.206	0.058	0.067	0.017

1

Supplementary material 2

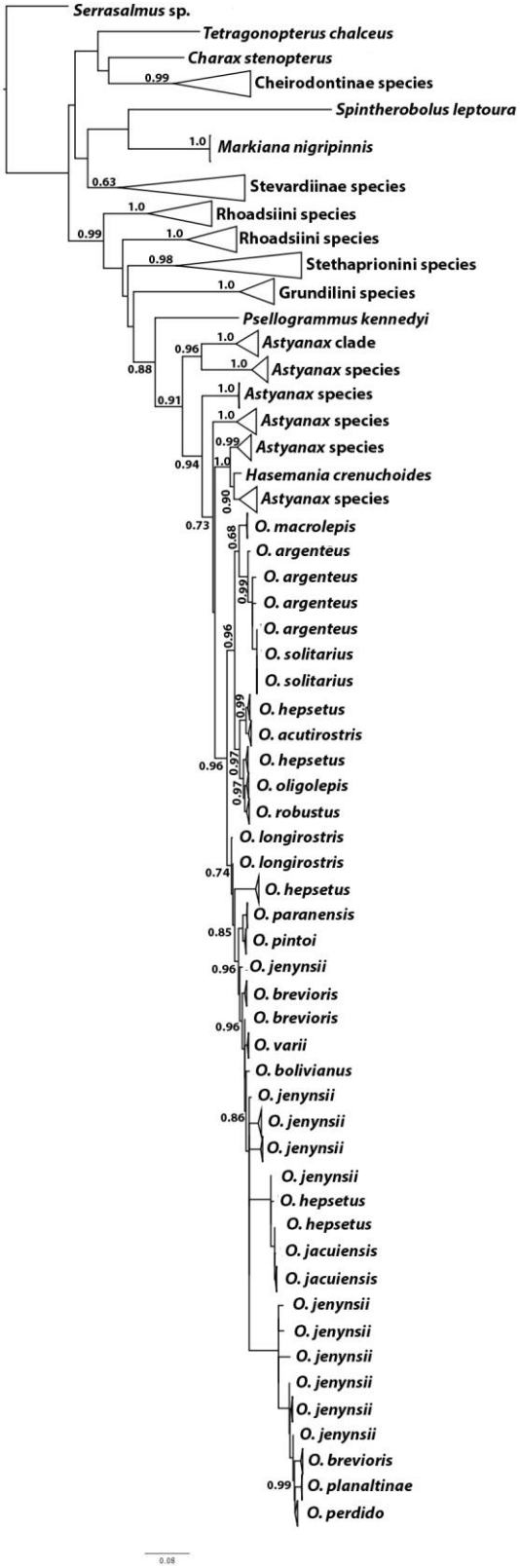


Fig. S1. Bayesian phylogenetic analysis of *Oligosarcus* and related genera based on the mitochondrial gene Cytochrome Oxidase C subunit 1 (COI). Posterior probabilities represented by values at the bases of the nodes. Posterior probabilities below 0.5 were not pictured in the phylogeny.

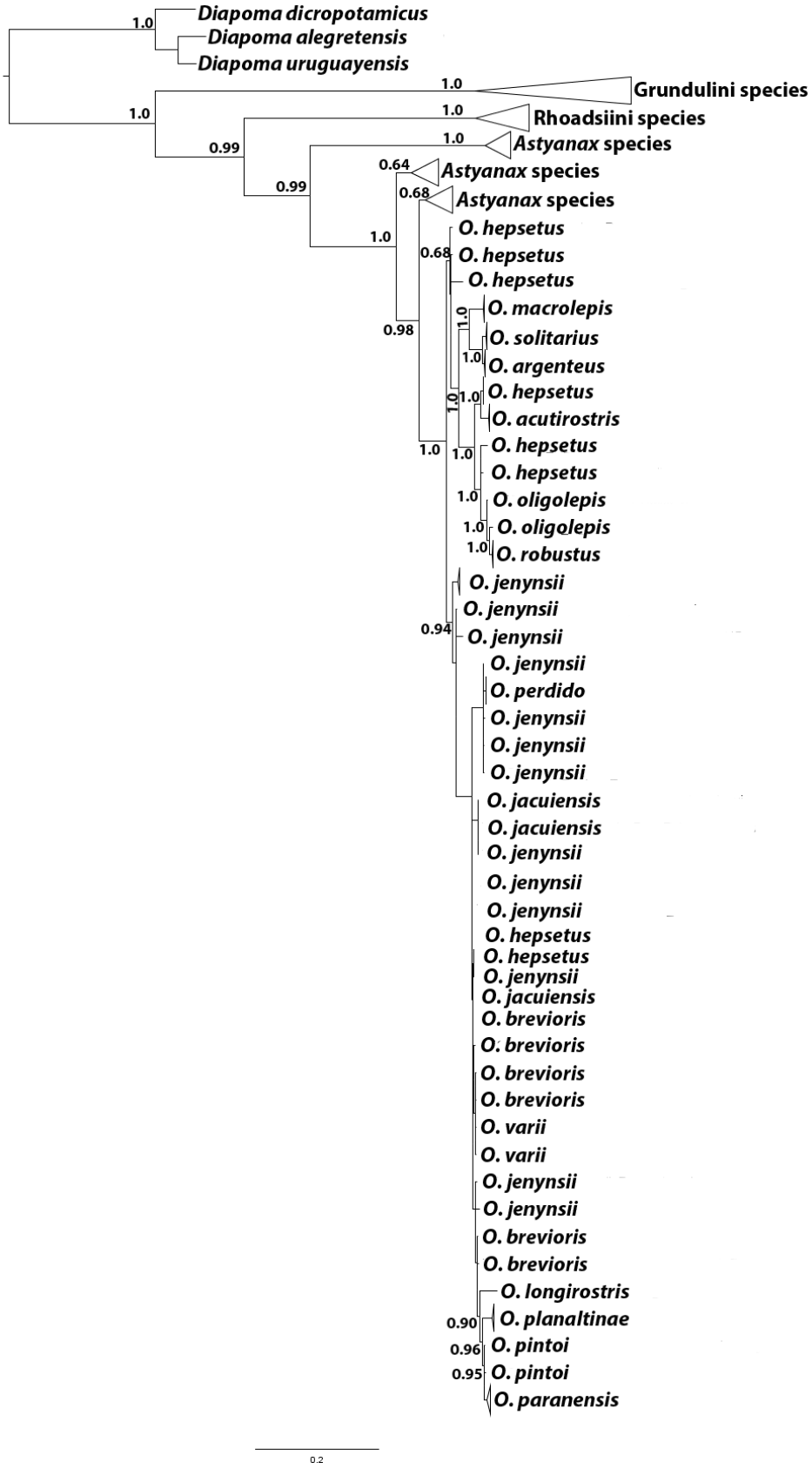


Fig. S2. Bayesian phylogenetic analysis of *Oligosarcus* and related genera based on the mitochondrial gene NADH dehydrogenase 2 (ND2). Posterior probabilities represented by values at the bases of the nodes. Posterior probabilities below 0.5 were not pictured in the phylogeny.

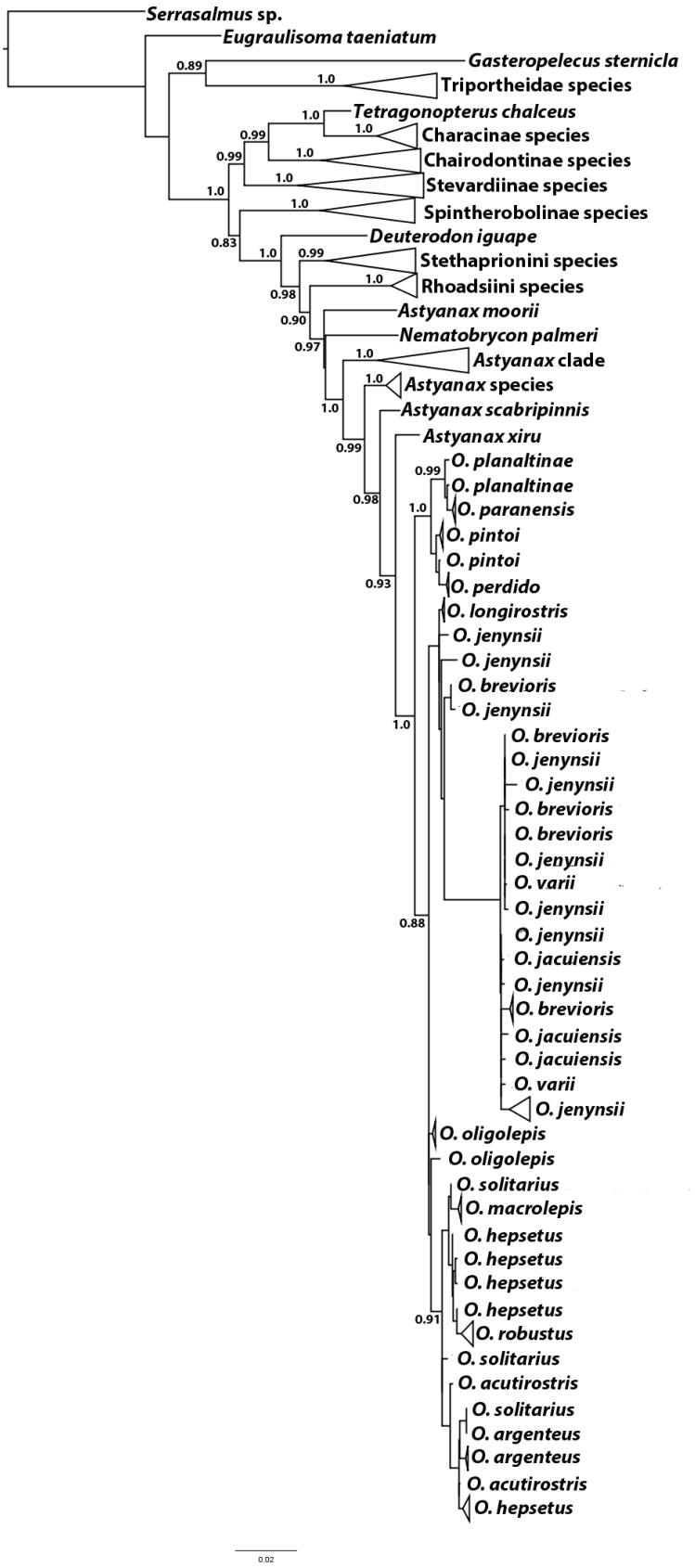


Fig. S3. Bayesian phylogenetic analysis of *Oligosarcus* and related genera based on the nuclear gene Recombination-Activating gene 2 (RAG2). Posterior probabilities by values at the bases of the nodes. Posterior probabilities below 0.5 were not pictured in the phylogeny.

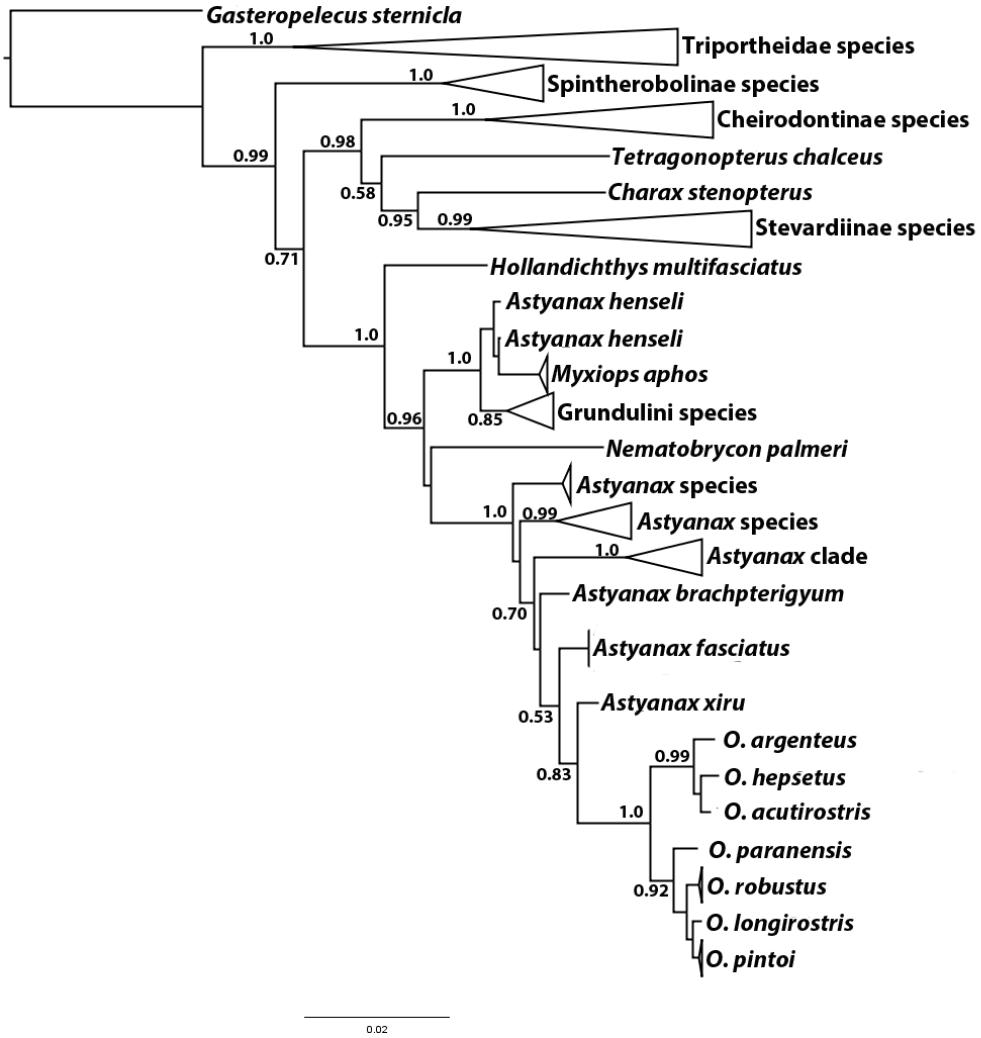


Fig. S4. Bayesian phylogenetic analysis of *Oligosarcus* and related genera based on the nuclear gene MYH6. Posterior probabilities represented by values at the bases of the nodes. Posterior probabilities below 0.5 were not pictured in the phylogeny.

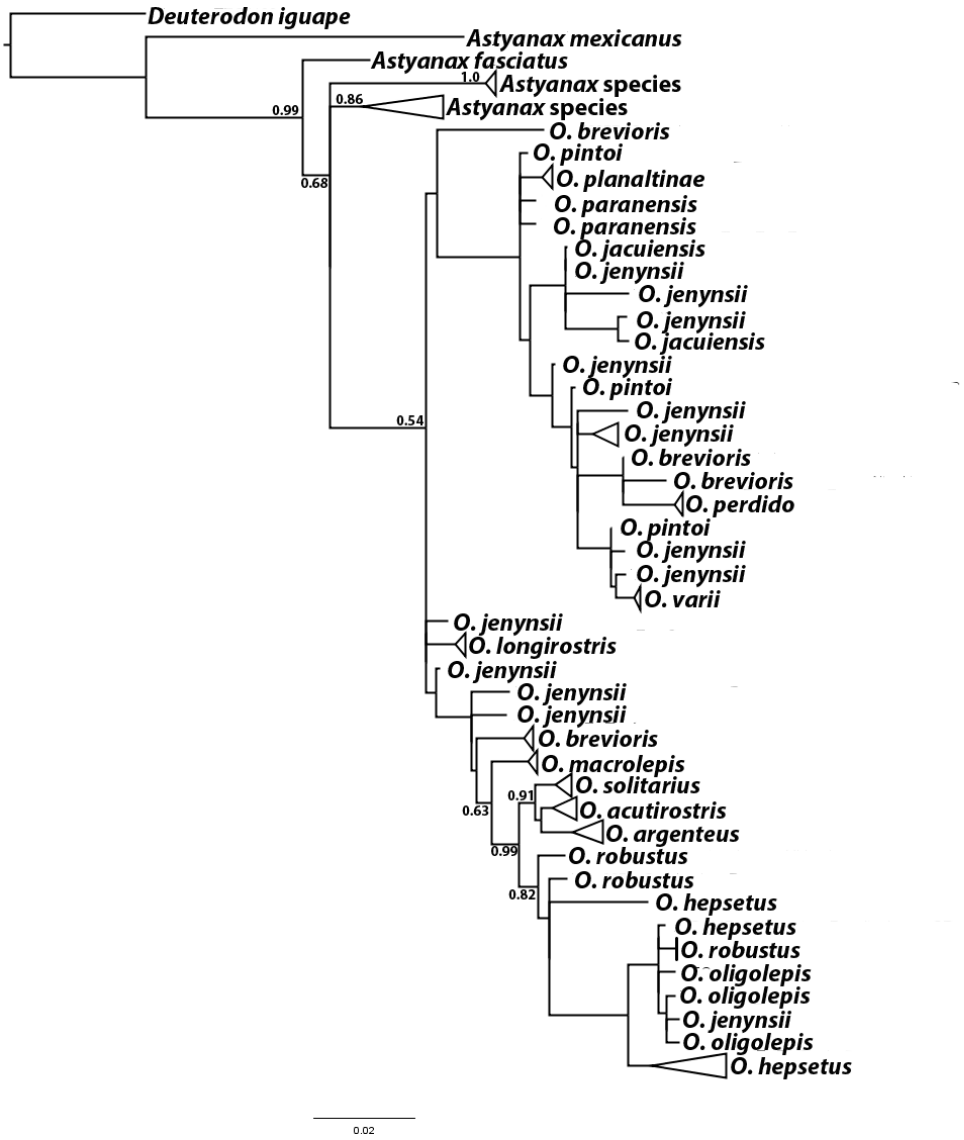


Fig. S5. Bayesian phylogenetic analysis of *Oligosarcus* and related genera based on the nuclear gene S7. Posterior probabilities represented by values at the base of nodes. Posterior probabilities below 0.5 were not pictured in the phylogeny.

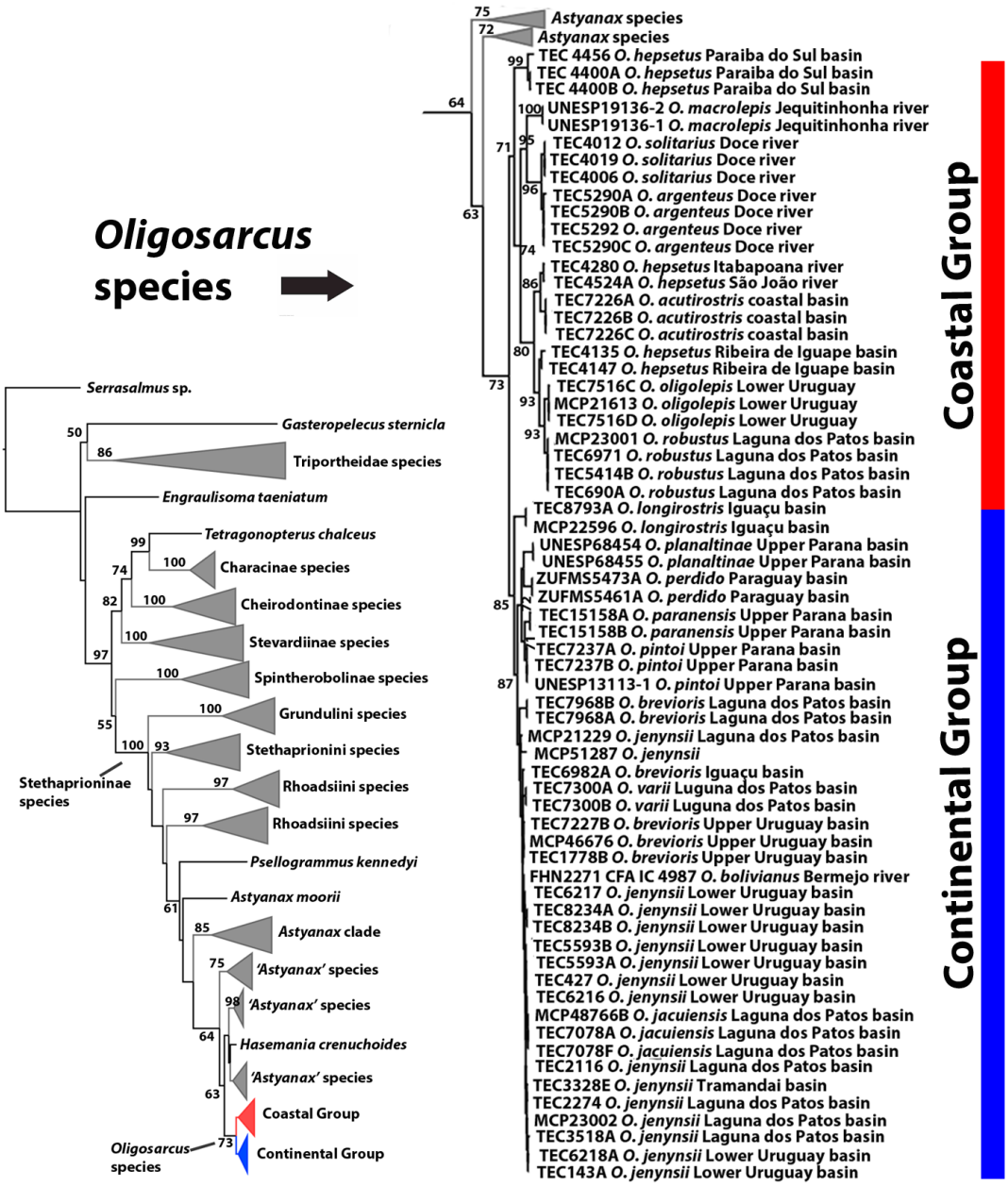


Fig. S6. Phylogenetic relationships within *Oligosarcus* species and outgroups based on Maximum Likelihood, using concatenated dataset. Branch support represented by values at the bases of the nodes. Bootstrap below 50 were not pictured in the phylogeny. A short descriptor of the locality follows species name.

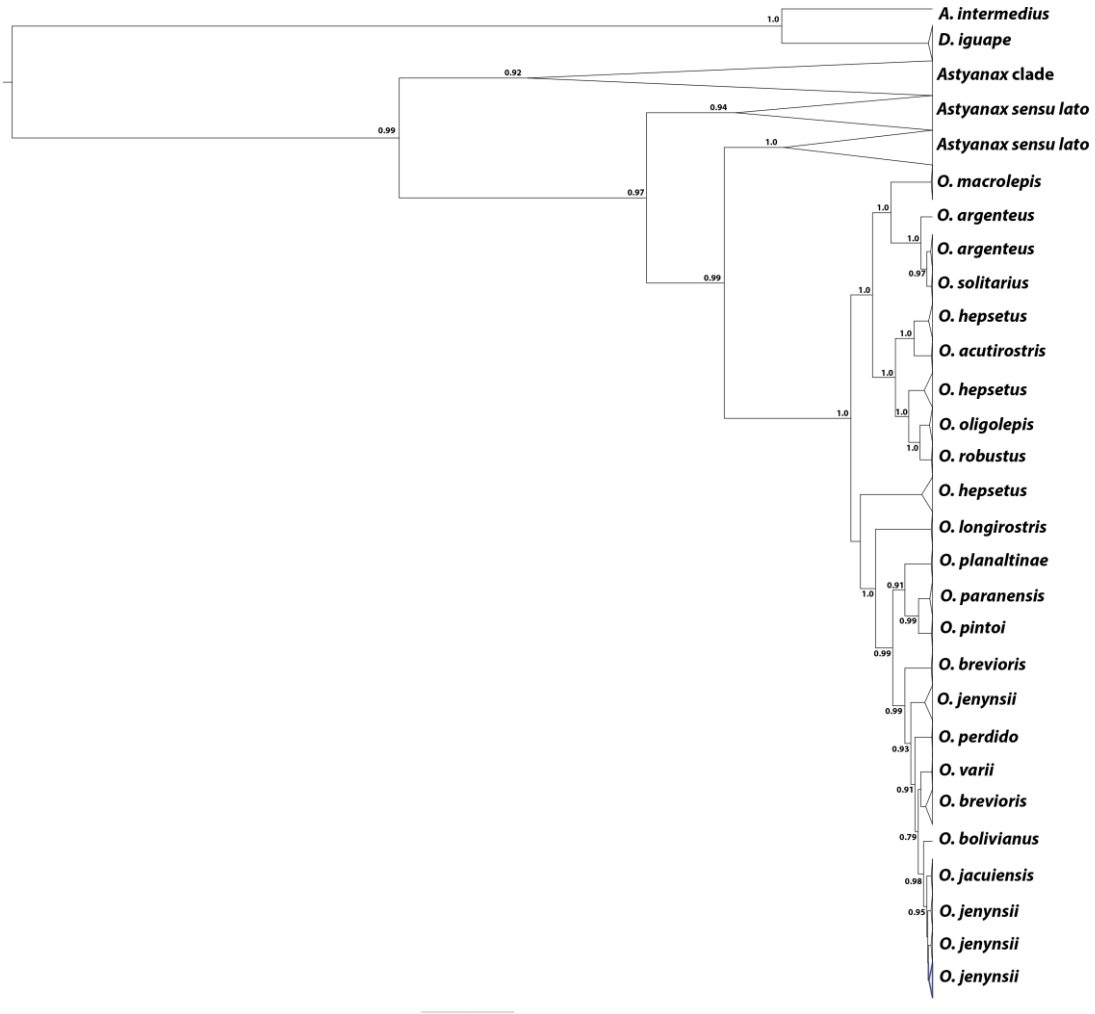


Fig. S7. Gene tree of *Oligosarcus* inferred by StarBEAST and based on the mitochondrial partition (COI + ND2). Posterior probabilities represented by values at the bases of the nodes. Posterior probabilities below 0.5 were not pictured in the phylogeny.

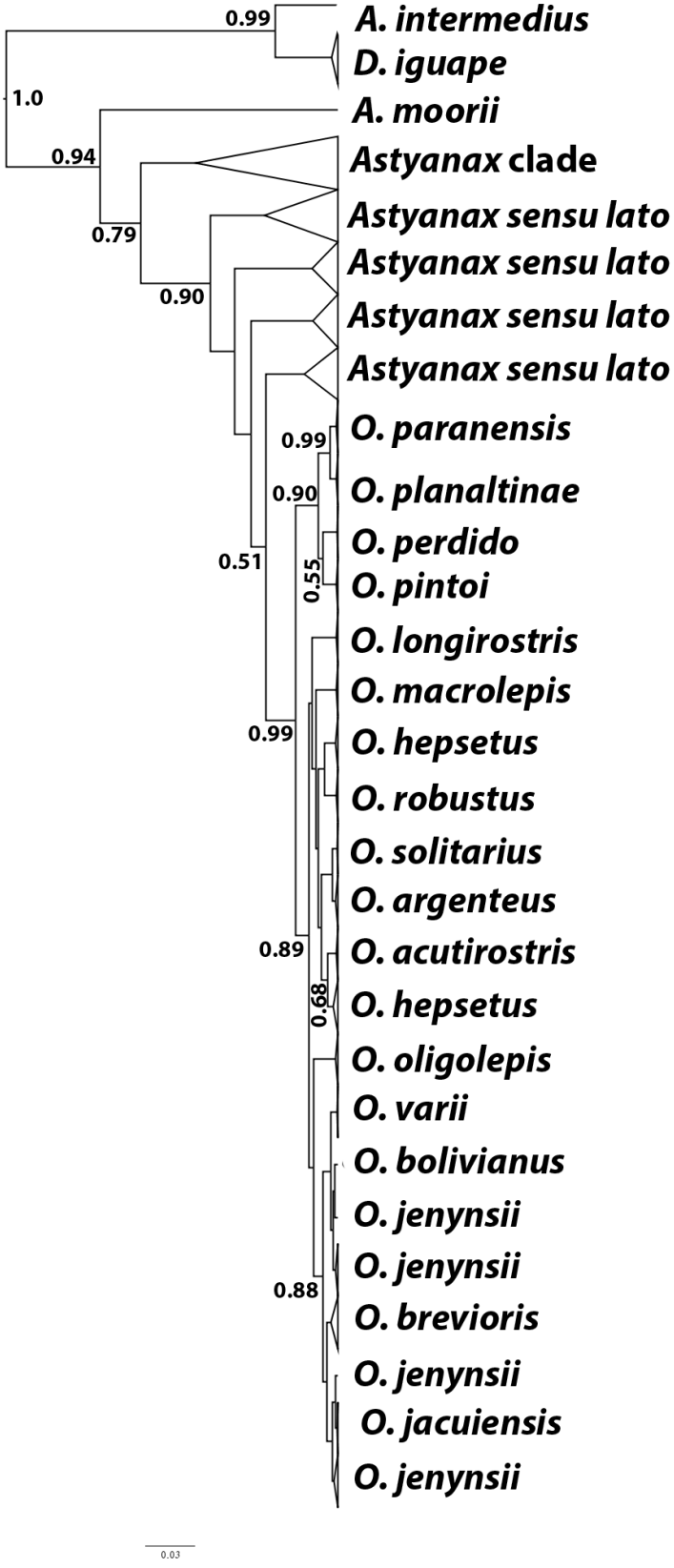


Fig. S8. Gene tree of *Oligosarcus* and related groups inferred by StarBEAST based on the nuclear gene Recombination-Activating gene 2 (RAG2). Posterior probabilities represented by values at the bases of the nodes. Posterior probabilities of nodes below 0.5 were not included in the phylogeny.

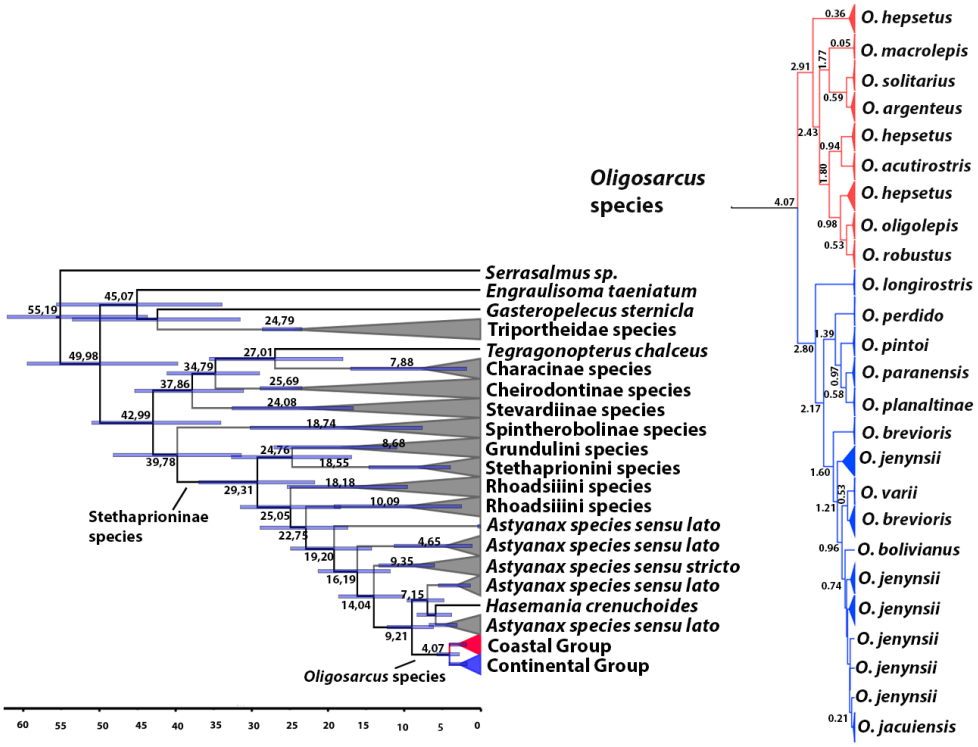


Fig. S9: Time-calibrated phylogeny of *Oligosarcus* species and outgroup. Fossils calibrations: †*Oligosarcus* sp. (node composed of *O. oligolepis* + *O. robustus*, prior age= 150 Ka); †*Paleotetra* (prior age= 33.9 Ma), †*M. unicus*, and †*L. ligniticus* (both with prior age= 23.03 Ma). Medium age (in millions of year) represented by values at the bases of the nodes. Scales bar represent time variation in millions of years.

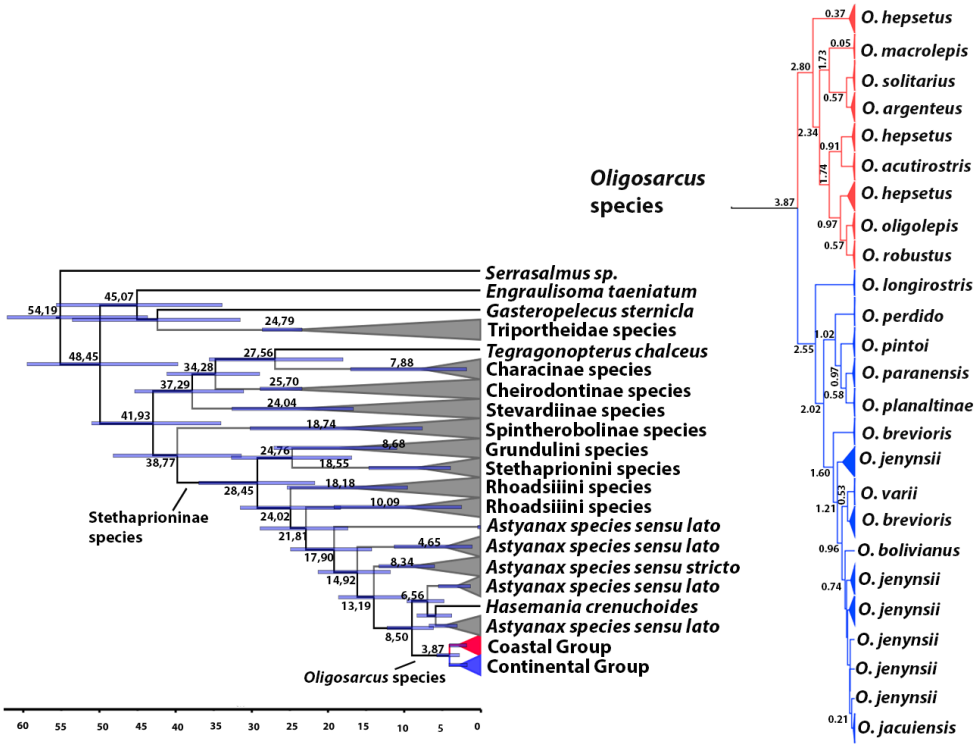
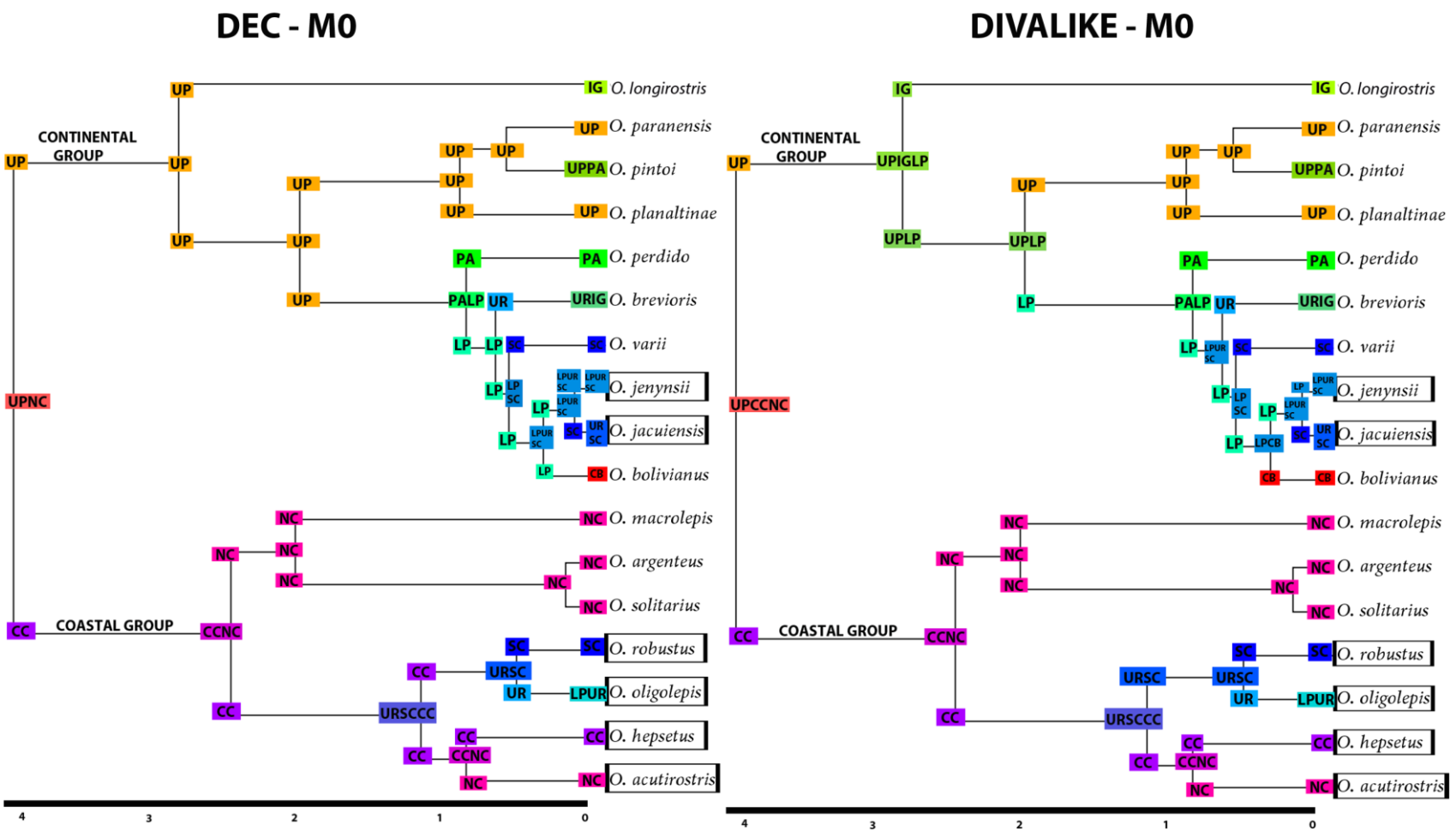
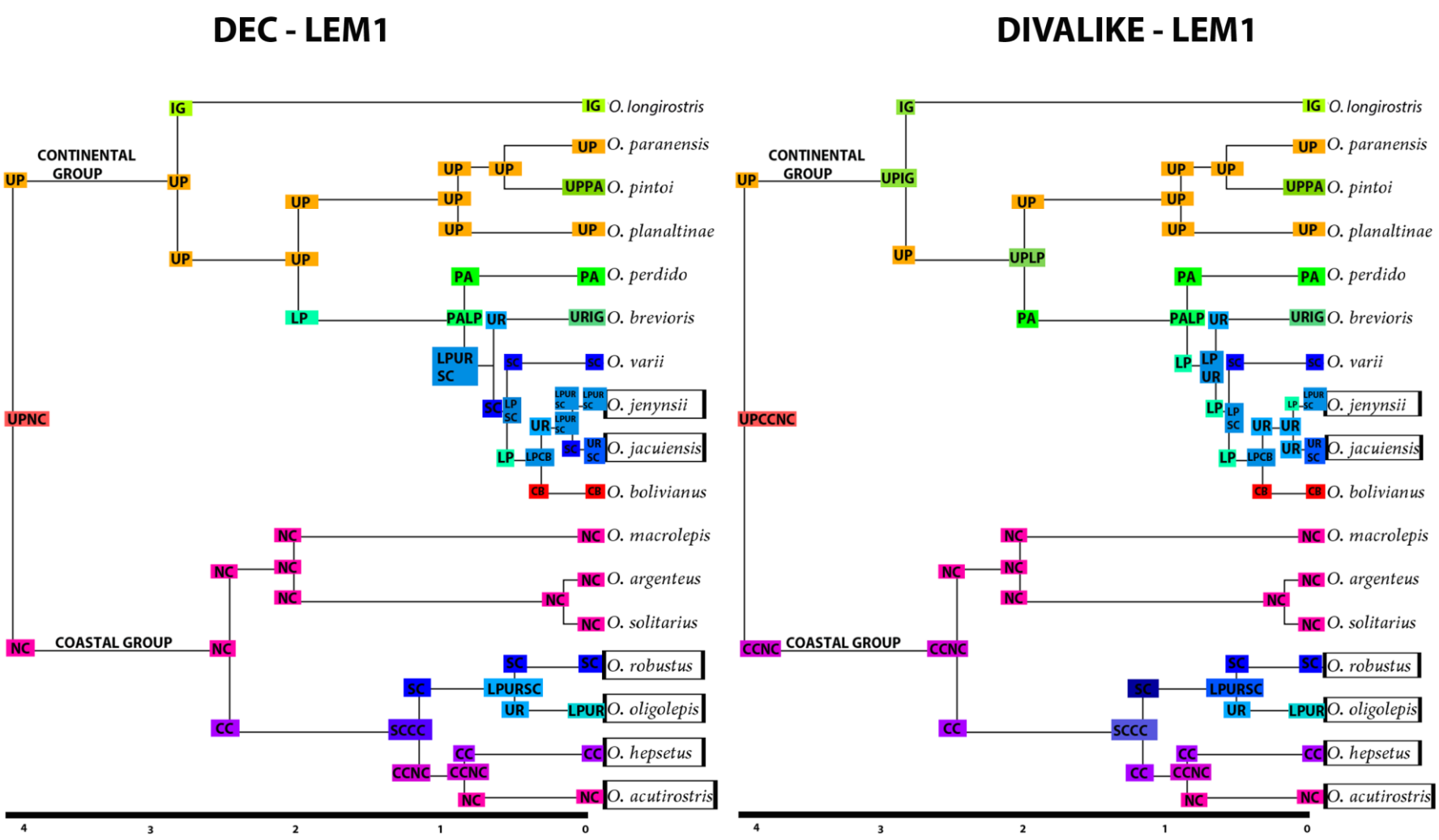
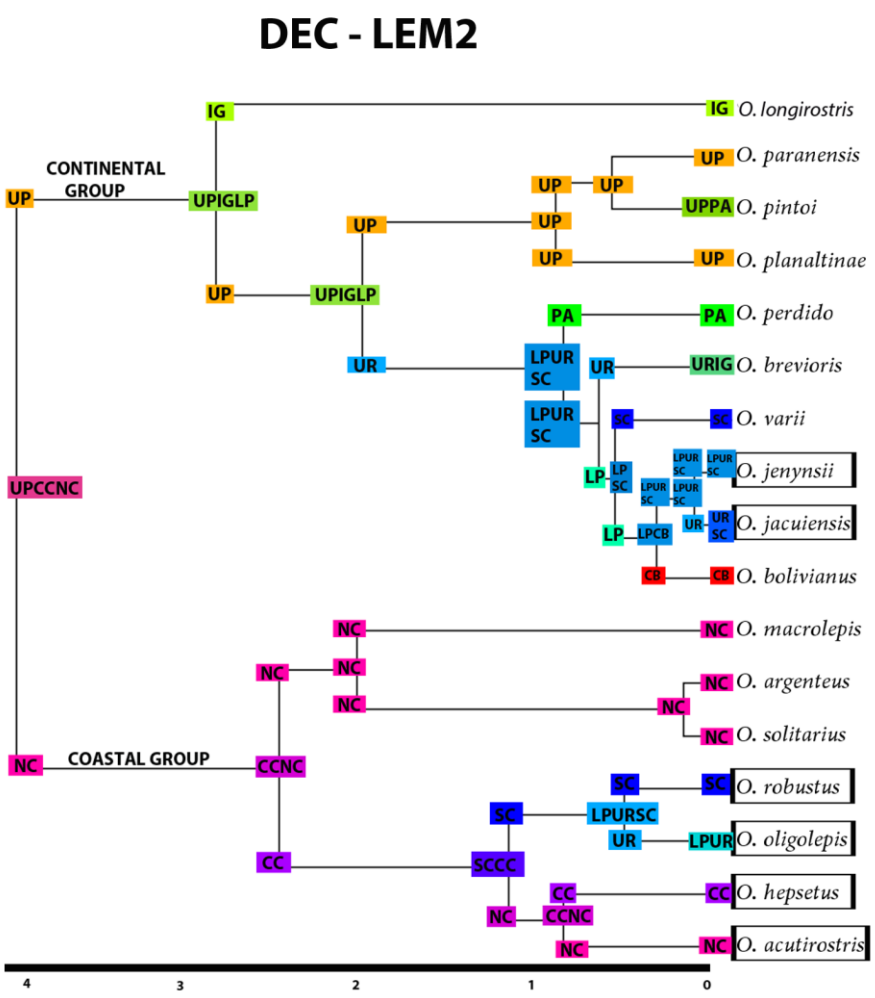


Fig. S10: Time-calibrated phylogeny of *Oligosarcus* species and outgroup. Fossils calibrations: †*Oligosarcus* sp. (node composed of *O. jenynsii* and close related species, prior age= 150 Ka); †*Paleotetra* (prior age= 33.9 Ma), †*M. unicus*, and †*L. ligniticus* (both with prior age= 23.03 Ma). Medium age (in millions of year) represented by values at the bases of the nodes. Scales bar represent time variation in millions of years.


DEC - M0
DIVALIKE - M0
CONTINENTAL GROUP
COASTAL GROUP
O. longirostris
O. paranensis
O. pintoi
O. planaltinae
O. perdido
O. brevioris
O. varii
O. jenynsii
O. jacuiensis
O. bolivianus
O. macrolepis
O. argenteus
O. solitarius
O. robustus
O. oligolepis
O. hepsetus
O. acutirostris
UPNC
UPCCNC
UPIGLP
UPLP
4
3
2
1
0


DEC - LEM1
DIVALIKE - LEM1
CONTINENTAL GROUP
COASTAL GROUP
O. longirostris
O. paranensis
O. pintoi
O. planaltinae
O. perdido
O. brevioris
O. varii
O. jenynsii
O. jacuiensis
O. bolivianus
O. macrolepis
O. argenteus
O. solitarius
O. robustus
O. oligolepis
O. hepsetus
O. acutirostris
4
3
2
1
0


DEC - LEM2
CONTINENTAL GROUP
COASTAL GROUP
O. longirostris
O. paranensis
O. pintoi
O. planaltinae
O. perdido
O. brevioris
O. varii
O. jenynsii
O. jacuiensis
O. bolivianus
O. macrolepis
O. argenteus
O. solitarius
O. robustus
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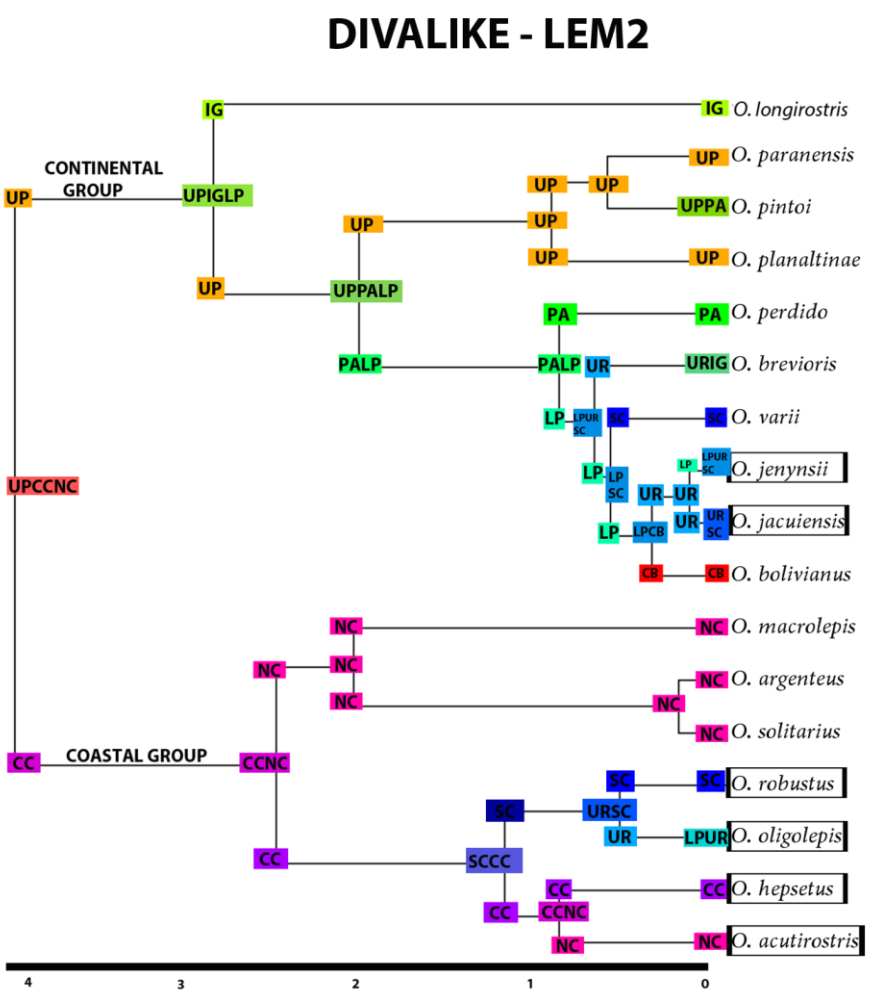
O. hepsetus
O. acutirostris
4
3
2
1
0


DIVALIKE - LEM2
CONTINENTAL GROUP
COASTAL GROUP
O. longirostris
O. paranensis
O. pintoi
O. planaltinae
O. perdido
O. brevioris
O. varii
O. jenynsii
O. jacuiensis
O. bolivianus
O. macrolepis
O. argenteus
O. solitarius
O. robustus
O. oligolepis
O. hepsetus
O. acutirostris
4
3
2
1
0

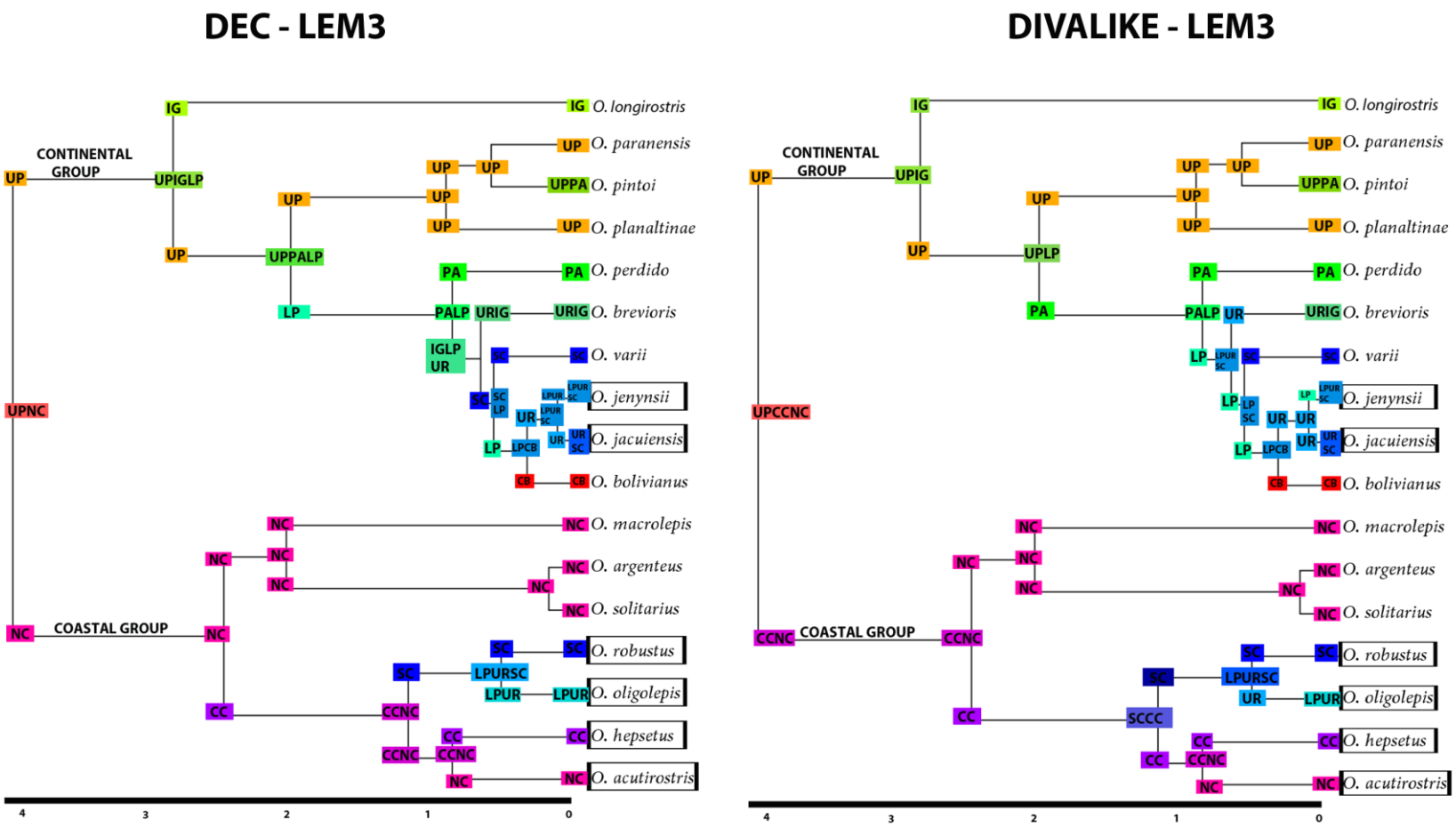
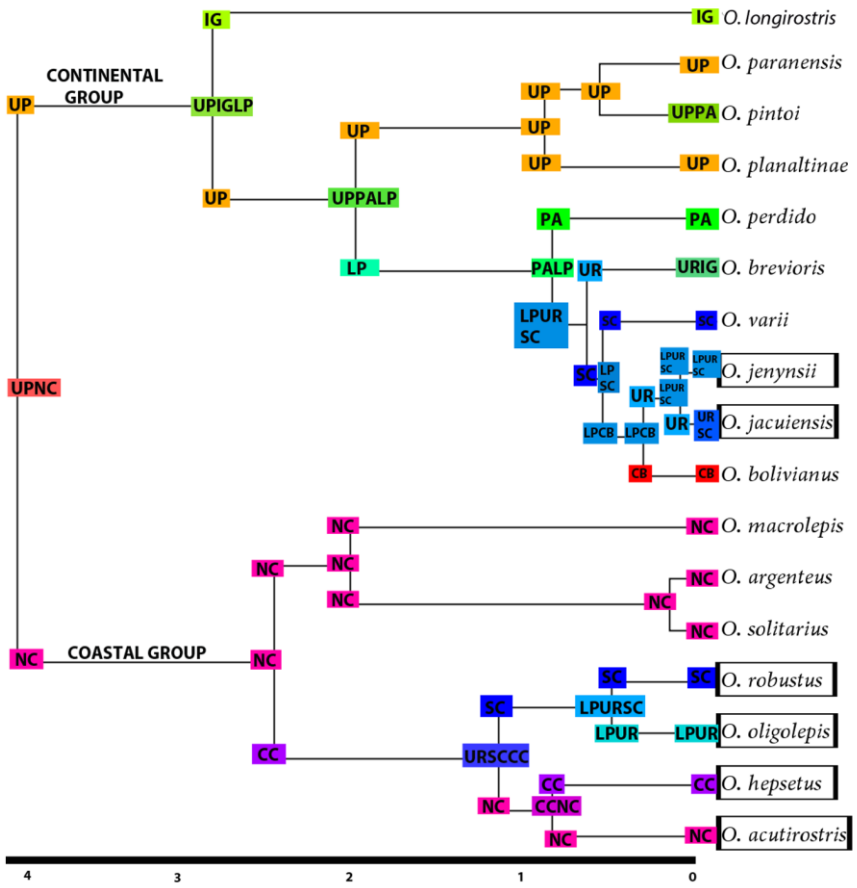


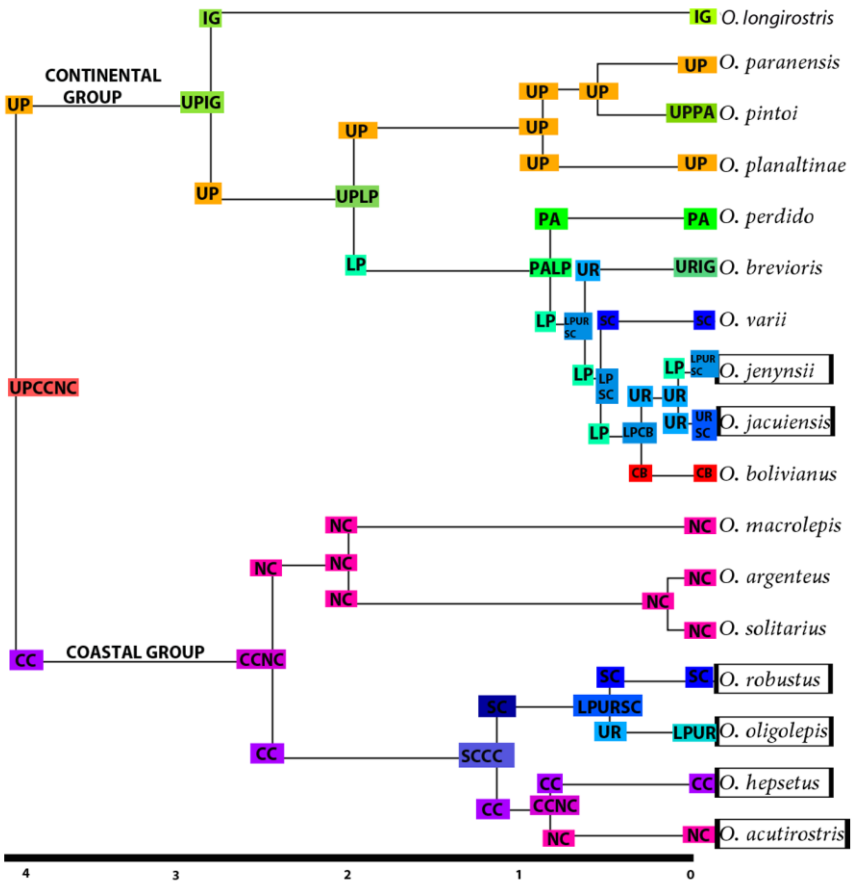
Fig. S11. Ancestral range estimation of *Oligosarcus* for the null model (M0) and Landscape evolution models (LEM's 1, 2 and 3) using DEC and DIVALIKE models, with quasi-impermeable dispersal rates (=0.1). Geographic units: CB=Chaco, PA=Paraguay, UP=Upper

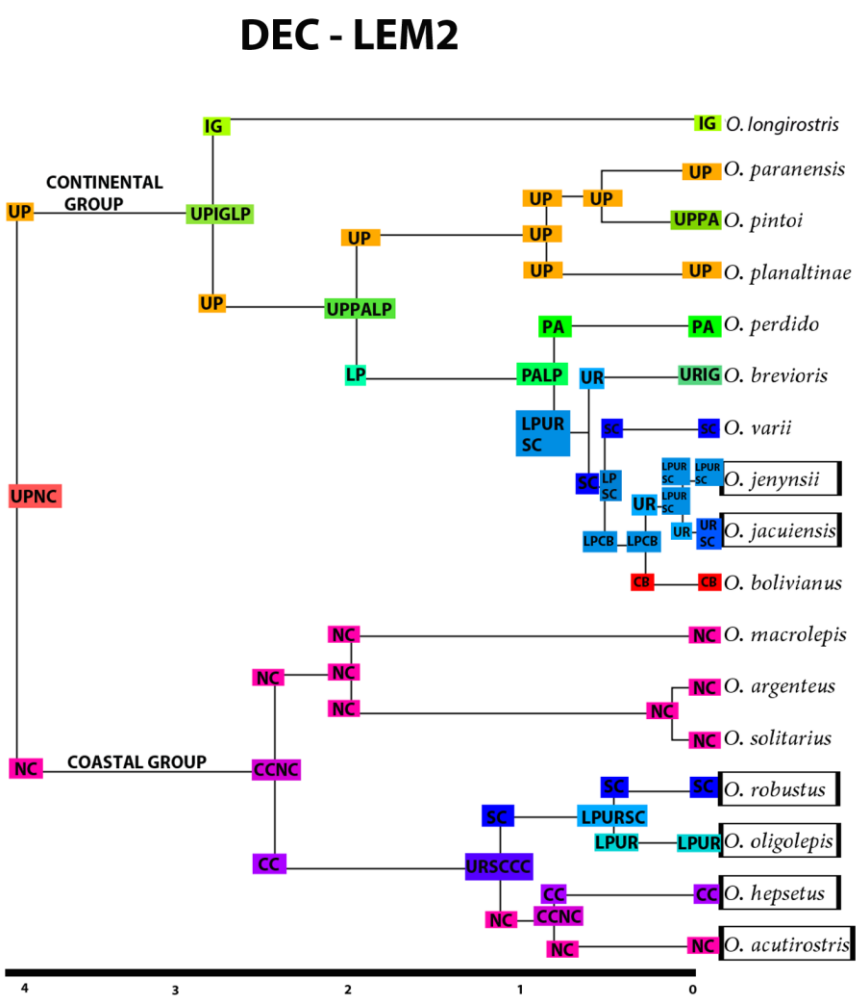
Paraná, LP=Lower Paraná, IG=Iguaçu, UR= Upper and Lower Uruguay ecoregions, NC=North Coastal, CC= Central Coastal, SC= South Coastal). Black rectangle surround species distribution in lowland areas (following Ribeiro & Menezes, 2015).

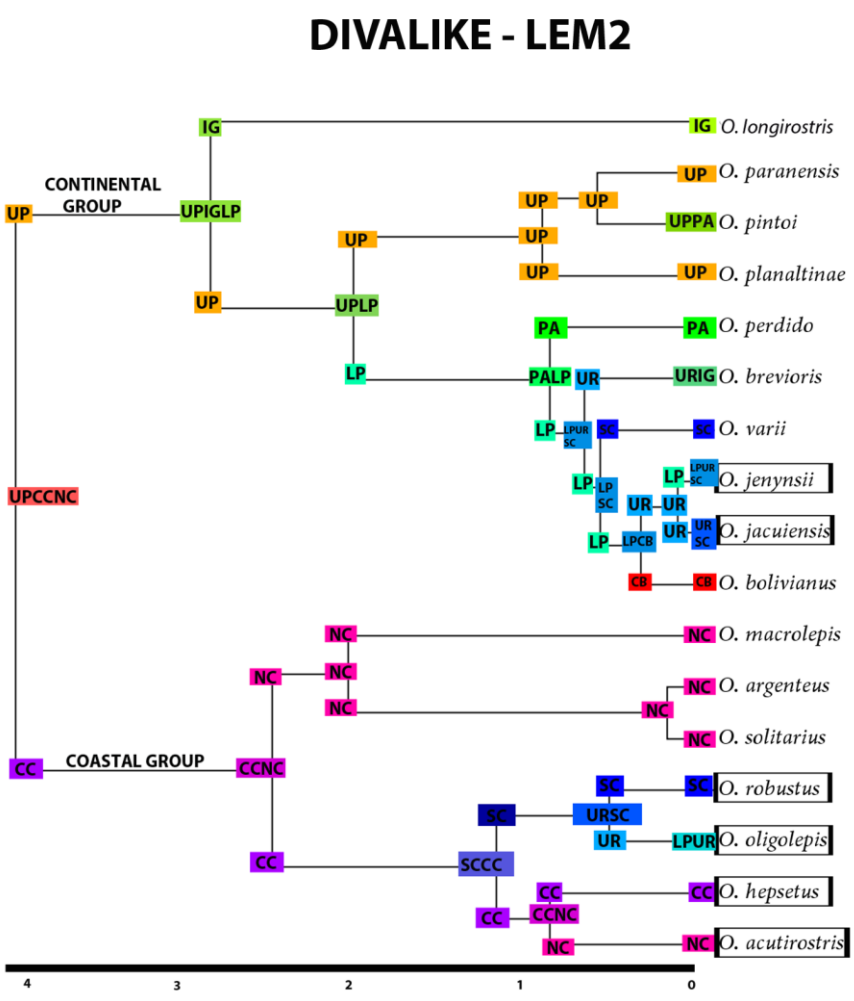
DEC - LEM1



DIVALIKE - LEM1




DEC - LEM2
CONTINENTAL GROUP
COASTAL GROUP
O. longirostris
O. paranensis
O. pintoi
O. planaltinae
O. perdido
O. brevioris
O. varii
O. jenynsii
O. jacuiensis
O. bolivianus
O. macrolepis
O. argenteus
O. solitarius
O. robustus
O. oligolepis
O. hepsetus
O. acutirostris
4
3
2
1
0


DIVALIKE - LEM2
CONTINENTAL GROUP
COASTAL GROUP
O. longirostris
O. paranensis
O. pintoi
O. planaltinae
O. perdido
O. brevioris
O. varii
O. jenynsii
O. jacuiensis
O. bolivianus
O. macrolepis
O. argenteus
O. solitarius
O. robustus
O. oligolepis
O. hepsetus
O. acutirostris
4
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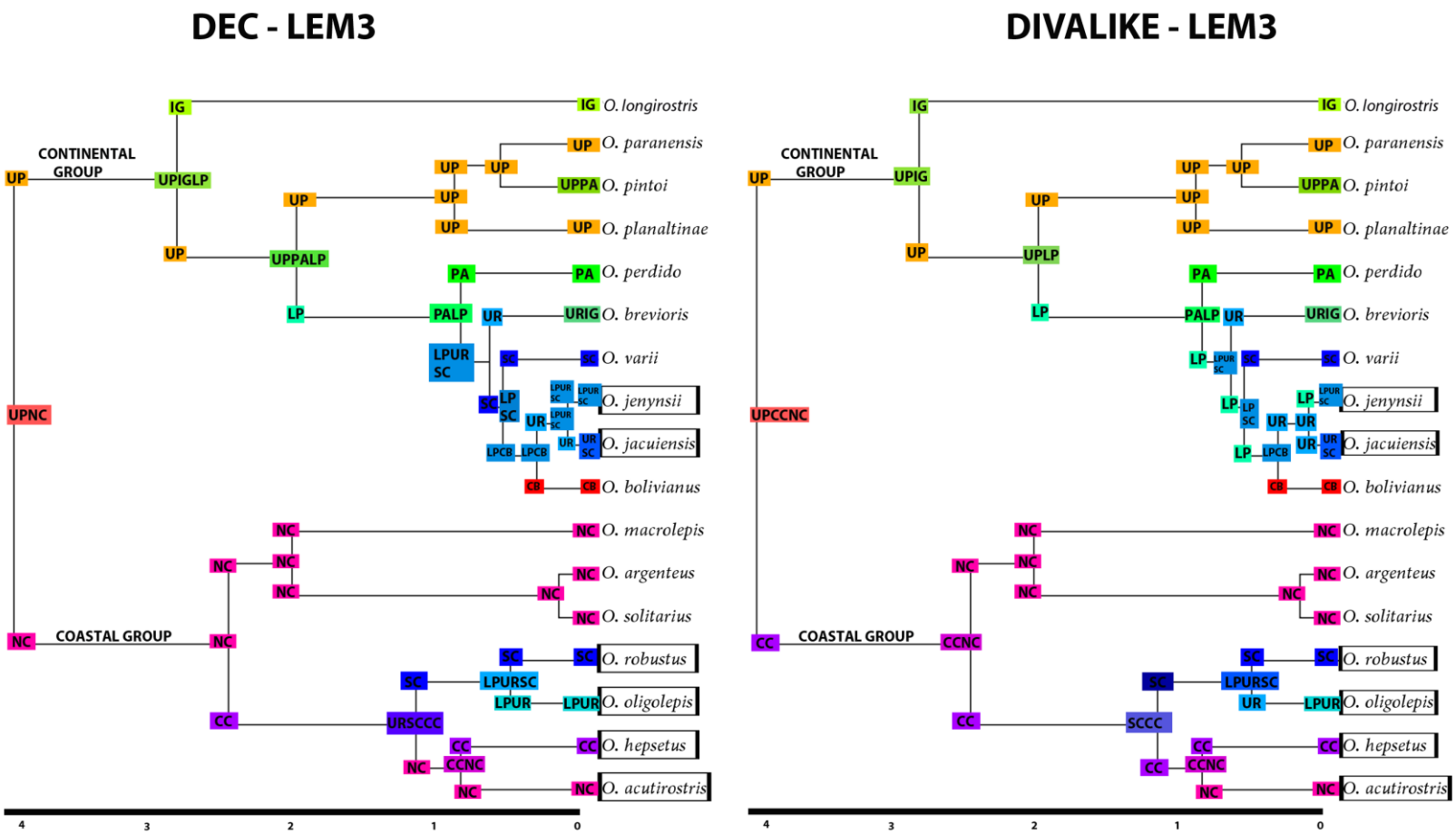

Fig. S12. Ancestral range estimation of *Oligosarcus* for the Landscape evolution models (LEM's 1, 2 and 3) using DEC and DIVALIKE models, with semi-permeable dispersal rates (=0.5). Geographic units: CB=Chaco, PA=Paraguay, UP=Upper Paraná, LP=Lower Paraná, IG=Iguaçu, UR= Upper and Lower Uruguay ecoregions, NC=North Coastal, CC= Central Coastal, SC= South Coastal). Black rectangle surround species distribution in lowland areas (following Ribeiro & Menezes, 2015).

CAPÍTULO II

Phylogeny, species limits and ecological and morphological diversity of *Characithecium* (Monogenoidea: Dactylogyridae): first insights into its host-parasite associations

Pretensão de submissão:
Parasitology ISSN: 0031-1820 (Print), 1469-8161 (Online)

Cap. 2 - Intention to publish on Parasitology

Phylogeny, species limits and ecological and morphological diversity of *Characithecium* (Monogenoidea: Dactylogyridae): first insights into its host-parasite associations

Emília W. Wendt[1*], Luiz R. Malabarba[1], Mariana P. Braga[2], Walter Boeger[3], Michael Landis[2], Tiago P. Carvalho[1,4]

[1]Laboratório de Ictiologia, Departamento de Zoologia, Universidade Federal do Rio Grande do Sul - UFRGS, Av. Bento Gonçalves, 9500, Bloco 4, prédio 43435, 91501-970 Porto Alegre, RS, Brazil.

[2]Department of Biology, Washington University in St. Louis, St. Louis, MO, 63130, USA

[3]Laboratório de Ecologia Molecular e Parasitologia Evolutiva (LEMPE), Departamento de Zoologia, Centro Politécnico, Setor de Ciências Biológicas, Universidade Federal do Paraná (UFPR), Caixa Postal 19073, CEP 81531-980, Curitiba, Paraná, Brazil.

[4]Laboratorio de Ictiología, Unidad de Ecología y Sistemática (UNESIS), Departamento de Biología, Facultad de Ciencias, Pontificia Universidad Javeriana, Carrera 7 N° 43-82, Bogotá D.C., Colombia.

* Corresponding author

Abstract

Characithecium is a monogenoid genus with seven species described from *Astyanax* and *Oligosarcus* host species in South and Central America. Previous proposals suggest a tight coevolutionary history between these parasites and their hosts, mainly due to the phylogenetic proximity of these genera of fish. To evaluate *Characithecium* diversity and its association with their hosts, we estimate phylogenetic relationships and divergence times, including all seven known species in a broad host spectrum, where *Characithecium* was recovered as monophyletic, having evolved at approximately 14 Ma. Then, we perform species diversity using a coalescent based GMYC and bPTP analyses which suggest fewer species than the morphological delimitation, recovered four and six entities respectively. Besides, our study expands the known geographical and host distribution for *Characithecium*, and test what kind of ecological traits (host species, ecoregion distributions, altitude, and type of water) are linked to the occurrence of *Characithecium* species. In general, this genus showed higher prevalences in *Oligosarcus* species than in *Astyanax*, being two species exclusive to *Oligosarcus*. Also, we used diagnostic

morphological data for the genus and conducted an ancestral character reconstruction in order to test if morphological characters evolved by descendency or are convergent in distinct species of *Characithecium*. In this case, two of the ten characters analyzed demonstrated to evolve by convergence, both associated with the structure and shape of the ventral bar. On the other hand, structures related to the male copulatory organ (MCO), accessory piece (AP) and hooks have been shown to evolve by descendency. The use of all these tools provided great knowledge about the evolutionary history within *Characithecium.*

Keywords: molecular relationships, divergence time, ancestral character-state.

Introduction

The reconstruction of the evolutionary history of parasites has been increasingly studied using host-parasite relationships and coevolutionary analyses. Monogenoidea Bychowsky, 1937 is a group of obligate parasites that is commonly found in freshwater fishes (Boeger and Vianna, 2006; Cohen *et al*., 2013), and is commonly used in studies that investigate host-parasite evolution (Domingues and Boeger, 2005; Mendlová and Šimková, 2014; Braga et al., 2015; da Graça et al., 2018). Monogenoidea parasitizing freshwater fishes is an excellent system to investigate host-parasite history due to the high host specificity (Boeger and Kritsky, 1993; 1997) and the geographic isolation of the hosts in hydrographic basins that have complex and reticulated biogeographical histories in South America (Albert and Reis, 2011). However, this type of study is often difficult to perform due to several reasons: (1) the difficulty in accurately delimitate species and (2) the high diversity of these parasites; as well as (3) the high diversity and (4) wide distribution of hosts, which combined make it difficult to collect and study these organisms.

Characithecium Mendoza-Franco, Reina, & Torchin, 2009 is a genus of monogenoids with seven species described, that parasitize gills of fishes distributed in freshwater habitats in Central (Mexico and Panama) and South America (Colombia, Brazil, and Argentina), being recorded so far exclusively on a few species of *Astyanax* [*Astyanax aeneus* (Gunther, 1860), *Astyanax ruberrimus* Eigenmann, 1913, *Astyanax fasciatus* (Cuvier, 1819), *Astyanax lacustris* (Lutken, 1875) and *Astyanax scabripinnis* (Jenyns, 1842)], and *Oligosarcus jenynsii* (Günther, 1864) (Kritsky and Leiby, 1972; Gioia *et al*., 1988; Boeger and Vianna, 2006; Gallas et al., 2016).

The genus was proposed to include one species that were, until then, classified as *Urocleidoides incertae sedis*, and parasitize gills of *Astyanax* species distributed from Mexico to Panama (Mendoza-Franco *et al*., 2009). In this study, Mendoza-Franco *et al* (2009) propose *Urocleidoides costaricensis* (Price and Bussing, 1967) as the type species of the new genus *Characithecium*. This genus remained monotypic until Rossin and Timi (2015) propose a diagnostic amendment for the genus and a new combination to accommodate *Palombitrema chascomusensis* Suriano, 1981 (= junior synonym of *C. chascomusensis*). Besides that, these authors described four new species found in gills of *O. jenynsii* collected in Chascomús Lake and Nahuel Rucá Lake, in the province of Buenos Aires (Argentina), in the south of South America. Later, Gallas *et al*. (2016) described its seventh species, *Characithecium triprolatum* Gallas, Calegaro-Marques and Amato, 2016, collected in gills of *A.* aff. *fasciatus* and *Astyanax jacuhiensis* Cope, 1894 [= junior synonym of *A. lacustris*] from Guaíba Lake, in the southernmost state of Brazil (Rio Grande do Sul).

The diagnostic characteristics for the genus are: (1) presence of articulation between male copulatory organ (MCO) and accessory piece (AP), (2) MCO tubular with spiral shaped in counterclockwise direction, (3) ventral anchor larger than dorsal anchor (Mendoza-Franco *et al*., 2009), and (4) the shape of the accessory piece which was described by Rossin and Timi (2015) as having 2 subunits forming a clamp-shaped piece. However, most of these diagnostic characteristics are found in several other genera of monogenoids in the Neotropical region, and should be reevaluated under a phylogenetic framework. Therefore, although the species diversity is relatively small, there is no relationship hypothesis for *Characithecium* species (Mendoza-Franco *et al*., 2009, Rossin and Timi, 2015, Gallas *et al*., 2016).

Characithecium seems to be specialized on gills of *Oligosarcus* and *Astyanax* species, which led Rossin and Timi (2015) to highlight the possibility of a close relationship between the evolutionary history of these host genera and parasite species. For this hypothesis to be tested, it is necessary to resolve *Characithecium* species phylogeny, to know its host diversity and to understand the evolutionary history of the hosts. *Oligosarcus* and *Astyanax* are part of the same tribe in the subfamily Stethaprioninae, a species-rich clade within Characidae (Mirande, 2018). *Oligosarcus* is a monophyletic genus composed of 22 species, distributed in the southeastern portion of South America (Ribeiro and Menezes, 2015; Wendt *et al*., 2019). On the other hand, *Astyanax* has a greater species diversity, with over 200 species (Fricke et al., 2019), being

recovered as a polyphyletic genus, where the species are distributed in different clades of Characidae (Mirande, 2018). Recently, Wendt *et al*. (2019) studied the phylogenetic relationship, divergence times and biogeography of *Oligosarcus* species, including an extensive outgroup formed by several *Astyanax* species in which *Oligosarcus* is nested. *Oligosarcus* radiation origins occurred in the Brazilian crystalline shield in the Pliocene (~ 5Ma) and its biogeographical history is associated with Pleistocenic sea-level changes and formation of barriers (waterfalls) in the Paraná River basin (Wendt *et al*., 2019).

If both *Oligosarcus* and *Astyanax* are parasitized by *Characithecium* species, the hypothesis of an evolutionary link between these genera can be examined, since these hosts are closely related, a fact that can contribute to the occurrence and specificity of *Characithecium* species in these hosts. Therefore, our goals are to understand *Characithecium* distribution in space (both geography and hosts), to recover their evolutionary history, to examine the morphological characters evolution and to examine what factors determine the occurrence of this genus. For this, we conducted an extensive search of the presence of this genus in species of *Oligosarcus* and *Astyanax*, across a wide geographical area, and investigate which ecological factors are associated with the occurrence of *Characithecium* species. Then, we investigated the phylogenetic relationship and the species delimitation for *Characithecium* based on molecular characters and estimated the divergence time for the genus. Finally, we conducted an ancestral character reconstruction, in order to test if morphological characters evolved by descendency or are convergent in distinct species of *Characithecium.*

Material and Methods

2.1. Parasite sampling

Host specimens were sampled from ichthyological collections in the following institutions: Universidade Estadual Paulista, São José do Rio Preto (DZSJRP); Universidade Estadual Paulista, Botucatu (LBP); Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre (MCP); Museu de Zoologia, Universidade Estadual de Londrina, Londrina (MZUEL); Universidade Federal do Rio Grande do Sul, Porto Alegre (UFRGS); and Coleção Zoológica da Universidade Federal do Mato Grosso do Sul, Campo Grande (ZUFMS). Parasite found in specimens fixed and preserved in 96% alcohol were extracted for molecular analyses, whereas parasites found in specimens fixed in formalin 10% and preserved in 70% alcohol were used for morphological identification and to assemble permanent blades. Additionally, host specimens were recently collected in field expeditions to fill gaps in geographic

distribution, being euthanized in clove oil (following Lucena et al., 2013) and then fixed and preserved in 96% alcohol. Collection permits of hosts were given by ICMBio to LRM. We examined parasite individuals from 351 specimens of *Oligosarcus* species and 124 specimens of *Astyanax* species were sampled from different populations in South America. Of these, 17 species of *Oligosarcus* were sampled, as well as 15 species of *Astyanax* close related to *Oligosarcus* (see Wendt *et al*. 2019), and often sympatric to this. The gills were intensively washed with 96% alcohol bursts using a syringe and whenever possible, all gill arches of the fish were carefully removed and analyzed. Then, parasite specimens were removed from the gills, stored in bottles containing 96% alcohol, and kept in a freezer at -4°C. Part of these parasite specimens (fixed in formalin 10%) were mounted on permanent blades using Hoyer, viewed in microscope Olympus BX51, and identified to the species level following morphological characteristics given by Mendoza-Franco *et al*. (2009), Rossin and Timi (2015) and Gallas *et al*. (2016). *Characithecium* species were determined on the basis of the size and shape of the sclerotized parts of the attachment organ (haptor) and the reproductive organs (Male Copulatory Organ-MCO and vaginal opening). Other parasite specimens (fixed in 96% alcohol) were used in molecular analysis, so they were mounted on temporary blade containing glycerin, identified to the species level and then one permanent blade was designated to represent this specimen, which served as a co-voucher for the samples used in molecular analyses. These co-vouchers were deposited in the CHIOC (Coleção Helmintológica do Instituto Oswaldo Cruz; Table S1). All applicable institutional guidelines for the care and use of animals were followed and approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil; CEUA-32283).

2.2. DNA extraction, PCR and sequencing

DNA was extracted from individual parasites (n = 36) according to the simplified method described by Tkach and Pawlowski (1999), in which it was built to provide minimal DNA material loss. Two ribosomal nuclear genes were amplified, 28S and 18S. The primers C1 (5'ACCCGCTGAATT TAAGCAT 3'), and C3 (5' CTCTTCAGAGTACTTTTCAAC 3') were used to amplify a fragment of approximately 400 bp of 28S (Mollaret *et al*. 2000). To amplify the 18S sequences, a set of primers (18S-188F and 18S-486R) was developed based on a sequence of *Diaphorocleidus armillatus* Jogunoori, Kritsky, and Venkatanarasaiah, 2004 (GenBank accession number

KT597997.1). For this, the software Primer3Plus (Untergasser *et al*., 2007) was used, and the quality of the primer was tested in the software NetPrimer (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html). Then, 18S-188F (5'TGACGTTGGATGTCAGACGG 3'), and 18S-486R (5' TAGTTTGTC TGGCGACGGTC 3') were used to amplify a fragment of approximately 460 bp of 18S. The PCR program for 28S was as follows: 5 min at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 45°C, 2 min at 72°C, and finally 7 min at 72°C. The PCR program for 18S was as follows: 5 min at 95°C, followed by 40 cycles of 1 min at 95°C, 45 s at 50°C, 1 min at 72°C, and finally 5 min at 72°C. Each amplification reaction contained 3-5 µl of template DNA, 3 mM MgCl2, 1X PCR-Buffer (Invitrogen), 0.5 pmol each primer, 0.4 mM dNTP and 1 U Platinum Taq polymerase (Invitrogen) in a total volume of 25 µl. PCR products were checked by electrophoresis in agarose gel, purified using ExoSap (Exonuclease I and Shrimp Alkaline Phosphatase GE Healthcare®, Piscataway, NJ, USA) and sequenced in both directions by ACTGene (Porto Alegre, Brazil). Forward and reverse sequences were visually inspected, edited, and combined into contigs using the software Geneious 8.0 (Kearse *et al*., 2012). The sequences of 28S and 18S of *Characithecium* species were deposited in GenBank (Table S1).

2.3. Phylogenetic reconstruction and species delimitation

Nucleotide substitution models to 28S and 18S genes were evaluated using PartitionFinder v1.1.1 (Lanfear *et al*., 2012; Table S2). Bayesian inference using BEAST2 v.2.4.5 (Bouckaert et al., 2014) was performed to estimate phylogenetic relationships of individual gene tree for 28S (the most densely sampled marker), for concatenated datasets, and for Species Tree analysis using both markers (28S and 18S). For 28S tree, the birth-death model was set as a tree prior and the relaxed clock log normal was configured as clock models and then, two runs of four chains were conducted simultaneously over 10,000,000 generations with sample frequency every 1,000 generations, where several species of Dactylogyridae family obtained from GenBank were used as outgroup. For concatenated analysis, we used both markers (28S and 18S), but just included *Characithecium* specimens and the genera *Jainus* and *Cacatuocotyle* as outgroups, as a way to reduce the numbers of missing data in the analysis since the 18S gene has a considerably smaller number of sequences. For this tree, the birth-death model was set as the tree prior and the strict clock was configured as clock models and then, we

performed the analysis with two runs of four chains, which were conducted simultaneously for 5,000,000 generations, with sample frequency every 500 generations.

For the Species Tree analysis, carried out using the StarBEAST 2.5 template (Heled and Drummond, 2010), we linked the 28S and 18S data sets. We performed the Species Tree analysis twice, where first we run without prior calibrations for date estimates on internal nodes, and later a Species Tree was generated again to include prior calibration dates on nodes based on divergence times estimation from the 28S dataset (see divergence time estimates analysis below). Morphologically delimited species were used as terminals as criteria for grouping specimens into putative species (Mendoza-Franco *et al*., 2009; Rossin and Timi, 2015; Gallas *et al*., 2016). Multi-species coalescence prior was set to constant root, and a tree model was set to the birth-death model with uniform distribution. The strict clock was configured as clock models. Priors for divergence time estimates were used on nodes under a normal distribution and were restricted to those nodes where the 28S time-calibrated tree was congruent with the first Species Tree analyses. Then, two runs of four chains were conducted simultaneously over 15,000,000 generations with sample frequency every 1,500 generations.

For all trees mentioned above, we inspected stationary posterior probabilities using Tracer v1.6 (Rambaut et al., 2014), and checked that the Effective Sample Size (ESS) of each parameter was above 200. Ten percent of the trees were discarded as burn-in. The remaining trees were used to compute a summary tree using the maximum clade credibility tree function with TreeAnnotator 2.4.3 (Bouckaert *et al*., 2014). All these analyses were implemented by XSEDE (3.2.6) in the CIPRES portal (Miller *et al*., 2010).

Finally, the molecular species-delimitation analyses were performed using the generalized mixed-yule coalescent (GMYC) method (Pons et al., 2006; Fujisawa and Barraclough, 2013), and the bayesian implementation of the Poisson Tree Processes (bPTP) method (Zhang et al., 2013). According to Zhang et al. (2013), these two methods of species delimitation differ significantly because GMYC uses time to identify branching rate transition points, while bPTP uses the number of substitutions. For the GMYC species delimitation method, we used the summarized ultrametric tree reconstructed using the 28S gene in BEAST2 v.2.4.5 (see above), and the analysis was performed in the R package "Splits" (Ezard et al. 2009) with a single threshold. The bPTP analysis was done in the online server (https://species.h-its.org/) using the unrooted tree, following the default parameters (with 100000 generations), and using summarized not ultrametric tree reconstructed using 28S gene performed in MrBayes 3.2.2. (Ronquist et al., 2012). For

this tree, we set K80+G as the nucleotide substitution model (as proposed by PartitionFinder) and performed two simultaneous runs of four chains over 10,000,000 generations with sample frequency every 1,000 generations.

2.4. Divergence time estimation

We performed a molecular time divergence analysis in BEAST v.2.5.1 (Bouckaert *et al*., 2014), using the 28S sequences for all taxa included in Table S1. For that, we used the evolutionary rate of the 28S proposed by three families and eleven genus within Proseriata (Platyhelminthes; Scarpa *et al*., 2015). A relaxed lognormal clock model was set, with an evolutionary rate of 0,005 mutations per million years for the 28S. The Birth-Death model was used as a tree prior (Heath *et al*., 2014). The analysis was performed with 20 million generations with sampled trees every 2.000 generations. Stationarity and sufficient mixing of parameters (ESS > 200) were checked using Tracer 1.6.

2.5. Occurrence and ecological traits of *Characithecium*

After collection and subsequent taxonomical identification of parasites, we characterized *Characithecium* species on: (1) host species (with number of fish specimens analyzed); (2) prevalence in each host species, i.e. the percentage of examined specimens that contained the focal parasite species; (3) parasite geographic distribution, which includes country, state, river basin, freshwater ecoregion and if it belongs to a coastal and/or a continental basins; (4) altitude of occurrence (in meters); and (5) categorical habitat type (river, stream, lagoon or a combination of them).

Then, we tested whether the prevalence (= frequency of occurrence) of each species of *Characithecium* is associated with some of the variables above. Generalized linear models (GLM) were used for this analysis, with a binomial distribution. First, 13 models were created (M1 to M13) with interactions between one or more of the following four variables: (1) geographic distribution - ecoregion, (2) habitat type, (3) altitude class, and (4) host species (Tab. 1; Fig. 1). For GLM analysis, the altitude values were transformed into 5 classes, based on the data distribution (class 1= 0 to 100 meters, class 2= 101 to 400 meters, class 3= 401 to 800 meters, class 4= 801 to 1200 meters, class 5= more than 1201 meters), and the ecoregion followed the Freshwater Ecoregions of the World (FEOW) proposed by Abell et al. (2008). In addition, we tested the null model (M0), where the frequency of the parasite species was not associated with any of the above variables. We used the Akaike's information criterion (AICc) to select the model(s) that

best explained the patterns, where the models with $\Delta AICc \leq 2$ were considered viable to explain the observed patterns (Burnham and Anderson, 2002). Lastly, we applied ANOVA to test the significance and obtained p-values ($p \leq 0.05$) for each best model(s).

Table 1. Models created with ecological variables used to explain the parasite frequency from GLM analysis.

Model	Variables included	Number of variables
Mo	Null model	-
M1	host + altitude class + habitat type + ecoregion	4
M2	Host + altitude class + habitat type	3
M3	Host + altitude class	2
M4	Host	1
M5	Host + habitat type	2
M6	Host + ecoregion	2
M7	Altitude class + habitat type + ecoregion	3
M8	Altitude class + habitat type	2
M9	Altitude class + ecoregion	2
M10	Habitat type + ecoregion	2
M11	Altitude class	1
M12	Habitat type	1
M13	Ecoregion	1

Figure 1.

2.6. Ancestral character-state estimation

To investigate how morphological characters evolved, we performed ancestral state reconstructions (Figs. 4-5) of ten discrete morphological characters. These ten characters were chosen because they are diagnostic for *Characithecium* and are important for the separation of species within the genus. Four of them were associated with reproductive organs: (1) articulation between Male Copulatory Organ-MCO and Accessory Piece-AP (*0*-articulated*; 1*-not articulated), (2) shape of AP (*0*-clamp-shaped or pincer-shaped*; 1*-rod-shaped*; 2*- not definite shape, (3) number of rings in MCO (*0*-one turn or less*; 1*-two to four turns*; 2*-more than four turns), and (4) the position of the vaginal opening (*0*-ventral; *1*- marginal). The other six characters are related to the fixation organ (haptor): (1) hook shank (*0*-none par dilated; *1*-some pairs, but not all, of dilated hooks; *2*-all 7 pairs dilated), (2) hooks size (*0*-all pairs with same size; *1*- pairs 1 and 5 smaller than pairs 2, 3, 4, 6 and 7; *2*-pairs 1, 5 and 7 larger than pairs 2, 3, 4 and 6), (3) size comparison between ventral and dorsal anchors (*0*-similar size, with dorsal anchor more than 70% of ventral anchor in size; *1*-different size, with length of dorsal anchor 70% or less than

ventral anchor in size), (4) posteromedian projection in ventral bar (*0*-absent; *1*-present), (5) medial suture in ventral bar (*0*- absent; *1*- present), and (6) ventral bar shape (*0*-straight or U-shape, *1*- V-shaped; *2*-not definite shape). All these characters were based on the original description of the species proposed by Mendoza-Franco et al. (2009), Rossin and Timi (2015), and Gallas et al. (2016), and on the specimens collected and observed in the present study.

The ancestral character state estimates was done using a maximum likelihood approach with three separate models of discrete character transitions (ER-equal rates, SYM – symmetrical and ARD – all rates different) performed using the ape package in R (Paradis et al., 2004). Stochastic character mapping of character was performed with the phytools package, also in R software (Revell, 2012). These analyses were done using the ultrametric tree estimated in the Species Tree analysis and the best model for each character was selected using the Akaike's information criterion (AICc).

Results

3.1. Phylogenetic relationships with divergence time estimation and species delimitations

Phylogenetic relationships of *Characithecium*, including all seven species, were estimated and presented here for the first time. The general characteristics of each gene are presented in Table S3. The 28S gene (~ 465bp) was amplified for a total of 38 individuals, while 18S gene, which corresponded to the largest region (~ 573bp), was successfully amplified for only 9 individuals. The K80+G model was used as nucleotide substitution model for 28S, while for 18S was used TrNef (Table S2). Within this sample universe, 28S showed a greater genetic variation than 18S, with 157 and 25 mutations, respectively (Table S3).

The phylogenetic relationships based on the 28S recovered *Characithecium* as monophyletic with high node support (PP= 0.98; Fig. 2). Also, the specimens were grouped in clades that supported the species identification (morphological identification), except for two species (*C. triprolatum* and *C. quadratum*). These two species were recovered in the same clade with high node support, but were not reciprocally monophyletic. In addition, the terminals within this clade (*C. triprolatum* + *C. quadratum*) showed significantly short branch sizes. Then we recovered a larger clade composed by other species of the genus, which was also recovered with high node support, being composed by *C. costaricensis* + (*C. longianchoratum* + (*C. chelatum* + (*C.

robustum + *C. chacomusensis*))). Species Tree analysis recovered the most node with high values, except the node composed by (*C. longianchoratum* + (*C. chelatum* + (*C. robustum* + *C. chacomusensis*))), with 0.51 PP (Fig. 3).

In the species delimitation analyses using molecular data (28 S) and coalescent based methods, the GMYC model recovered just four species, while bPTP model recovered six species (Fig. 3). Both methods recognized the species *C. triprolatum* and *C. quadratum* as belonging to the same taxonomical unit. In addition, GMYC did not recover *C. chelatum, C. robustum* and *C. chascomusensis* as distinct species, being all three nominal species recognized as a single unit. On the other hand, bPTP analysis showed that all remaining species (*C. costaricensis, C. longianchoratum, C. chelatum, C. robustum* and *C. chascomusensis*) form distinct taxonomical units (Fig. 3).

The divergence time estimations recovered that the origin of *Characithecium* diversification (its first cladogenetic event) is dated to approximately 14 Ma (95% HPD = 20.8–8.93 Ma). Also, was estimated that the clade *C. triprolatum* + *C. quadratum* would have an approximate date of 2.62 Ma (95% HPD = 4.6–1.25 Ma). Then, it was estimated that *C. costaricensis* would have diverged from the other species of the genus around 6.60 Ma (95% HPD = 9.95–3.92 Ma), and the divergence between *C. robustum* and *C. chascomusensis* was estimated at approximately 0.81 Ma (95% HPD = 1.65–0.22 Ma; Fig. 2).

Figure 2.

Figure 3.

3.2. Occurrence and ecological traits in *Characithecium*

We identified a large number of new hosts and expanded the geographic distribution for species of *Characithecium* (Tab. 2). With the new data presented in our study, we observe that *Characithecium* species, in general, are widely found in a large number of *Oligosarcus* species and, to some degree, in *Astyanax* species. Regarding the number of host species, *Characithecium* occurs in at least 32 fish species, with most interactions occurring at low prevalence rates (Tab. 2). In general, *Characithecium* showed a higher prevalence on *Oligosarcus* species than on *Astyanax* species (Tab. 2). *Oligosarcus bolivianus*, a fish species distributed in the Bermejo River basin, was the main host for four of the seven parasite species (*C. chascomusensis*, *C. chelatum, C. longianchoratum*, and *C. quadratum*), with prevalence rates between 75 to 100% (Tab. 2).

The species *C. longianchoratum* and *C. robustum* were found exclusively in *Oligosarcus* species (Tab. 2), the first being reported in seven host species, with higher prevalence in *O. bolivianus*, and considerably decreasing its prevalence in host species that have distribution in the southern region of South America (e.g. *O. jacuiensis, O. jenynsii*, and *O. varii*). On the other hand, *C. robustum* was found only in three species of *Oligosarcus*, having a higher occurrence in *O. longirostris*, in the Iguaçu River basin, followed by *O. jenynsii*, in the Uruguay and Laguna dos Patos basins (Tab. 2).

Different from the others, *C. costaricensis* was more frequent in *Astyanax* species (in seven species) and was only positive, but with low prevalence, for *O. hepsetus* collected in basins along the coastal region of Brazil, and for *O. macrolepis* collected in the Jequitinhonha River. On the other hand, *C. quadratum* was reported in five species of *Oligosarcus* and only in two species of *Astyanax*, presenting higher prevalence for *O. bolivianus* in the Bermejo River basin and species in Laguna dos Patos and Uruguay basins (Table 2).

The other species of *Characithecium* have a wide range of hosts, including *Oligosarcus* and *Astyanax* species (Tab. 2). *Characithecium chelatum*, was the most generalist species in terms of the number of hosts used (19 species), but it showed a significantly higher association in species with distribution in the Bermejo, Paraguay and La Plata river basins (Lower and Upper Paraná and Uruguay) (Tab. 2). *Characithecium triprolatum* was reported for 18 host species, with higher prevalence in *O. perdido* and *A.* aff. *fasciatus*, followed by other species with lower prevalence. Finally, *C. chascomusensis* was found in 15 host species, with *O. bolivianus* being the species with the highest prevalence, however, followed by species occurring in basins in the coastal region of Brazil (e.g. *O. robustus, O. solitarius, A. bagual* and *A. douradilho*).

Regarding the models tested by GLM (Tab. 1 and 3) evaluating the occurrence of *Characithecium* species, *C. costaricensis* has 80% of its occurrence explained by model 9, which took into account the altitude class and ecoregion variables. This model recovered higher prevalence values of *C. costaricensis* associated with high altitudes (class 4= 801 to 1200 meters) and in following ecoregions: São Francisco, Northeastern Mata Atlantica, Laguna dos Patos and Tramandaí-Mampituba (Tab. 3). Model 9 also explained 49% of the occurrence of *C. quadratum*, but in locations with low altitudes (0 to 100m) from the Laguna dos Patos. Still, *C. quadratum* had 25% of its occurrence explained by model 4, which concerns host species, being the high prevalence of this

parasite associated with *O. bolivianus, O. robustus, O. oligolepis* and *A. douradilho* (Tab. 3).

On the other hand, *C. robustum* had 95% of its occurrence explained by model 10, which took into account the habitat type and ecoregion variables, where this parasite was associated with rivers from Iguaçu, Uruguay and Laguna dos Patos ecoregions. The model 10 also explained the 75% of occurrence of *C. triprolatum* and *C. longianchoratum*. The first species was associated with rivers and lagoons from Laguna dos Patos, Tramandaí-Mampituba, Uruguay, and the other ecoregions of central coastal of Brazil. Differently, the occurrence of *C. longianchoratum* was associated with rivers and stream from Laguna dos Patos. Finally, the species *C. chelatum* and *C. chascomusensis* had their high prevalence explained by the fish species (model4 – with 84 and 43% respectively), the first being more prevalent in *O. bolivianus, A. brachypterygium, O. varii, O pintoi* and *O. paranensis*, and the second most prevalent in *O. bolivianus, O. robustus, O. solitarius, A. bagual,* and *A. douradilho* (Tab. 3).

403 **Table 2**. Species of ~~C~~*haracithecium* parasitizing gills of *Oligosarcus* and *Astyanax* species, with prevalence, ecology and geographic distribution of hosts in South and Central
404 America. m= meters. Coa=Coastal basins and Con=Continental basin according to Wendt et al. (2019). FEOW = Freshwater Ecoregions of the World according to Abell et al.
405 (2008).

Characithecium species	Host species (n° of specimens analyzed)	Parasite %	Country	State	Hydrographic basin	FEOW	Region	Altitude (m)	Water body type
***C. costaricensis* (type species) (Price & Bussing, 1967)**	*O. hepsetus* (34)	5,88% (2)	Brazil	Santa Catarina	Itajaí River	South Mata Atlantica	Coa	69	river
	O. macrolepis (9)	11,1% (1)	Brazil	Minas Gerais	Jequitinhonha River	Northeastern Mata Atlantica	Coa	723	river
	A. mexicanus (11)	36,36% (4)	Mexico	-	-	-		772	-
	A. rivularis (8)	12,5% (1)	Brazil	Minas Gerais	Preto River	São Francisco	Con	692	stream
	Astyanax sp. (8)	50% (4)	Brazil	Minas Gerais	Doce River	Northeastern Mata Atlantica	Coa	1175	stream
	A. eigenmanniorum (14)	7,14% (1)	Brazil	Rio Grande do Sul	Tramandaí River	Laguna dos Patos	Coa	12	lagoon
	A. xiru (8)	25% (2)	Brazil	Rio Grande do Sul	Ijuí and Maquiné rivers	Tramandaí-Mampituba, Lower Uruguay	Coa	54 to 127	River, stream
	A. cremnobates (10)	20% (2)	Brazil	Rio Grande do Sul	Upper Maquiné River	Tramandaí-Mampituba	Coa	870	River
	A. brachypterygium (6)	50% (3)	Brazil	Rio Grande do Sul	Taquari, and Pelotas rivers	Laguna dos Patos, Upper Uruguay	Coa and Con	1068 to 1181	River, stream
***C. chascomusensis* (Suriano, 1981)**	*O. acutirostris* (38)	21,05% (8)	Brazil	Bahia and Minas Gerais	Santo Antonio, and Mucuri rivers	Northeastern Mata Atlantica	Coa	41 to 174	river
	O. argenteus (36)	8,33% (3)	Brazil	Minas Gerais	Doce, and Upper São Francisco rivers	Northeastern Mata Atlantica, São Francisco	Coa and Con	663 to 980	River, lagoon

O. bolivianus (4)	75% (3)	Bolivia, Argentina	-	Bermejo River	Chaco	Con	609 to 2200	River, stream
O. brevioris (23)	21,7% (5)	Brazil	Rio Grande do Sul, Santa Catarina	Uruguai, and Canoas rivers	Upper Uruguay	Con	700 to 792	River, stream
O. hepsetus (34)	38,2% (13)	Brazil	São Paulo, Rio de Janeiro, Espírito Santo, Santa Catarina	Ribeira de Iguape and Itajaí Rivers. Coastal basins of Rio de Janeiro and Espirito Santo	Ribeira de Iguape, Northeastern Mata Atlantica, Fluminense, South Mata Atlantica	Coa	13 to 94	River, stream, lagoon
O. jacuiensis (22)	22,7% (5)	Brazil	Rio Grande do Sul	Upper Jacuí, and Taquari rivers	Laguna dos Patos	Coa	293 to 471	river
O. jenynsii (37)	24,3% (9)	Brazil	Rio Grande do Sul	Tramandaí River, Patos Laguna, and Ibicuí River	Laguna dos Patos, Lower Uruguay	Coa and Con	4 to 119	River, lagoon
O. longirostris (6)	16,6% (1)	Brazil	Paraná	Iguaçu River	Iguaçu	Con	229	river
O. paranaensis (13)	23% (3)	Brazil	Paraná	Piquiri River	Upper Paraná	Con	403	river
O. robustus (26)	61,5% (16)	Brazil	Rio Grande do Sul	Tramandaí River, Laguna dos Patos, Lagoa Mirim, 3 Forquilhas River, and Jacuí River	Laguna do Patos	Coa	2 to 133	River, lagoon
O. solitarius (13)	61,5% (8)	Brazil	Minas Gerais	Doce River	Northeastern Mata Atlantica	Coa	258	lagoon
O. varii (21)	4,76% (1)	Brazil	Rio Grande do Sul	São Marcos River	Laguna dos Patos	Coa	552	river
A. bagual (6)	50% (3)	Brazil	Rio Grande do Sul	Taquari River	Laguna dos Patos	Coa	179	river

	A. douradilho (6)	50% (3)	Brazil	Rio Grande do Sul	Maquiné River	Tramandaí-Mampituba	Coa	72	river
	A. henseli (10)	20% (2)	Brazil	Rio Grande do Sul	Upper Jacuí River	Laguna dos Patos	Coa	272	river
***C. chelatum* Rossin & Timi, 2015**	*O. argenteus* (36)	44,4% (16)	Brazil	Minas Gerais	Doce, and Upper São Francisco rivers	Northeastern Mata Atlantica, São Francisco	Coa and Con	606 to 980	River, stream, lagoon
	O. bolivianus (4)	100% (4)	Bolivia, Argentina	-	Bermejo River	Chaco	Con	609 to 2200	River, stream
	O. brevioris (23)	8,6% (2)	Brazil	Rio Grande do Sul, Santa Catarina	Uruguai and Canoas rivers	Upper Uruguay	Con	700 to 792	River, stream
	O. hepsetus (34)	8,8% (3)	Brazil	São Paulo, Santa Catarina	Ribeira de Iguape and Itajaí rivers	Ribeira de Iguape, South Mata Atlantica	Coa	42 to 69	River
	O. jacuiensis (22)	4,5% (1)	Brazil	Rio Grande do Sul	Upper Jacuí river	Laguna dos Patos	Coa	471	river
	O. jenynsii (37)	13,5% (5)	Brazil	Rio Grande do Sul	Caí, Tramandaí and Maquiné rivers	Laguna dos Patos	Coa	4 to 802	River, lagoon
	O. longirostris (6)	33,3% (2)	Brazil	Paraná	Iguaçu river	Iguaçu	Con	229	river
	O. oligolepis (14)	42,8% (6)	Brazil	Rio Grande do Sul	Uruguai, Ibicuí and Negro rivers	Lower Uruguay	Con	35 to 150	River, stream
	O. paranaensis (13)	53,8% (7)	Brazil	Paraná	Tabagí, Piquiri and Ivaí rivers	Upper Paraná	Con	403 to 572	river
	O. perdido (10)	40% (4)	Brazil	Mato Grasso do Sul	Perdido river	Paraguay	Com	456 to 519	River
	O. pintoi (18)	55,5% (10)	Brazil	São Paulo	Rio Grande river	Upper Paraná	Con	479	stream
	O. planaltinae (11)	54,5% (6)	Brazil	Distrito Federal	Paranaiba river	Upper Paraná	Con	870 to 939	stream

	O. varii (21)	80% (17)	Brazil	Rio Grande do Sul	São Marcos river	Laguna dos Patos	Coa	552 to 714	River, stream
	A. eigenmanniorum (14)	14,2% (2)	Brazil	Rio Grande do Sul	Tramandaí river	Laguna dos Patos	Coa	12	lagoon
	A. paranae (6)	16,6% (1)	Brazil	Minas Gerais	Paranaiba river	Upper Paraná	Con	761	river
	A. xiru (8)	37,5% (3)	Brazil	Rio Grande do Sul	Ijuí and Maquiné rivers	Tramandaí-Mampituba, Lower Uruguay	Coa and Con	17 to 197	stream
	A. cremnobates (10)	10% (1)	Brazil	Rio Grande do Sul	Upper Maquiné river	Tramandaí-Mampituba	Coa	870	River
	A. brachypterygium (6)	83,3% (5)	Brazil	Rio Grande do Sul	Taquari river	Laguna dos Patos	Coa	1068	river
	A. henseli (10)	10% (1)	Brazil	Rio Grande do Sul	Tramandaí river	Laguna dos Patos	Coa	5	lagoon
***C. longianchoratum* Rossin & Timi, 2015**	*O. bolivianus* (4)	75% (3)	Bolivia, Argentina	-	Bermejo river	Chaco	Con	609 to 2200	River, stream
	O. hepsetus (34)	17,6% (6)	Brazil	São Paulo, Rio de Janeiro	Ribeira de Iguape river and coastal basin of Rio de Janeiro	Ribeira de Iguape, Fluminense	Coa	18 to 59	River, stream
	O. jacuiensis (22)	9,09% (2)	Brazil	Rio Grande do Sul	Taquari river	Laguna dos Patos	Coa	862	river
	O. jenynsii (37)	21,6% (8)	Brazil	Rio Grande do Sul	Caí, Tramandaí, Jaguarão, Ibicuí and Uruguai rivers	Laguna dos Patos, Lower Uruguay	Coa and Con	4 to 790	River, stream, lagoon
	O. oligolepis (14)	28,5% (4)	Brazil	Rio Grande do Sul	Ibicuí and Negro rivers	Lower Uruguay	Con	110 to 150	River, stream
	O. paranaensis (13)	15,3% (2)	Brazil	Paraná	Piquiri rivers	Upper Paraná	Con	403	river
	O. robustus (26)	19,2% (5)	Brazil	Rio Grande do Sul	Tramandaí river and Laguna dos Patos	Laguna do Patos	Coa	4 to 12	lagoon

***C. quadratum* Rossin & Timi, 2015**	*O. bolivianus* (4)	75% (3)	Bolivia, Argentina	-	Bermejo river	Chaco	Con	609 to 2200	River, stream
	O. hepsetus (34)	2,9% (1)	Brazil	Santa Catarina	Itajaí river	South Mata Atlantica	Coa	69	River
	O. jenynsii (37)	13,5% (5)	Brazil	Rio Grande do Sul	Tramandaí, and Jaguarão rivers	Laguna dos Patos	Coa	1 to 132	stream, lagoon
	O. oligolepis (14)	42,8% (6)	Brazil	Rio Grande do Sul	Uruguai, Ibicuí and Negro rivers	Lower Uruguay	Con	35 to 150	River, stream
	O. robustus (26)	46,1% (12)	Brazil	Rio Grande do Sul	Jacuí, and Tramandaí river and Laguna dos Patos	Laguna do Patos	Coa	4 to 43	Stream, lagoon
	A. douradilho (6)	33,3% (2)	Brazil	Rio Grande do Sul	Lower Maquiné river	Tramandaí-Mampituba	Coa	65 to 147	River
	A. henseli (10)	10% (1)	Brazil	Rio Grande do Sul	Tramandaí river	Laguna dos Patos	Coa	5	river
***C. robustum* Rossin & Timi, 2015**	*O. jenynsii* (37)	32,4% (12)	Brazil	Rio Grande do Sul	Lagoa Mirim, Tramandaí, Ibicuí, and Rio Negro river	Laguna dos Patos, Lower Uruguay	Coa and Con	2 to 165	River, stream, lagoon
	O. longirostris (6)	83,3% (5)	Brazil	Paraná	Iguaçu river	Iguaçu	Con	229	river
	O. oligolepis (14)	7,14% (1)	Brazil	Rio Grande do Sul	Ibicuí river	Lower Uruguay	Con	101	river
***C. triprolatum* Gallas, Calegaro-Marques & Amato, 2016**	*O. acutirostris* (38)	13,15% (5)	Brazil	Bahia	Santo Antonio river	Northeastern Mata Atlantica	Coa	41	river
	O. argenteus (36)	30,5% (11)	Brazil	Minas Gerais	Doce, and Upper São Francisco rivers	Northeastern Mata Atlantica, São Francisco	Coa and Con	663 to 980	River, stream, lagoon
	O. brevioris (23)	13,04% (3)	Brazil	Rio Grande do Sul	Taquari river	Laguna dos Patos	Coa	1012	stream

O. hepsetus (34)	20,5% (7)	Brazil	São Paulo	Ribeira de Iguape	Ribeira de Iguape	Coa	42 to 94	River, lagoon
O. jacuiensis (22)	9,09% (2)	Brazil	Rio Grande do Sul	Taquari river	Laguna dos Patos	Coa	452	river
O. jenynsii (37)	10,8% (4)	Brazil	Rio Grande do Sul	Tramandaí, Jaguarão, and Ibicuí rivers	Laguna dos Patos, Lower Uruguay	Coa and Con	7 to 119	River, stream, lagoon
O. oligolepis (14)	14,28% (2)	Brazil	Rio Grande do Sul	Ibicuí and Negro rivers	Lower Uruguay	Con	110 to 140	stream
O. paranaensis (13)	23,07% (3)	Brazil	Paraná	Piquiri and Ivaí rivers	Upper Paraná	Con	403 to 572	river
O. perdido (10)	80% (8)	Brazil	Mato Grasso do Sul	Perdido river	Paraguay	Com	456 to 519	River
O. robustus (26)	15,3% (4)	Brazil	Rio Grande do Sul	Tramandaí, Mampituba and Maquiné rivers	Tramandaí-Manpituba, Laguna dos Patos	Coa	2 to 10	lagoon
O. varii (21)	4,7% (1)	Brazil	Rio Grande do Sul	São Marcos river	Laguna dos Patos	Coa	552	river
A. dissensus (6)	33,3% (2)	Brazil	Rio Grande do Sul	Tramandaí river	Laguna dos Patos	Coa	4	lagoon
A. aff. fasciatus (8)	62,5% (5)	Brazil	Rio Grande do Sul	Upper Jacui and Uruguai rivers, Guaíba lagoon	Laguna dos Patos, Lower Uruguay	Coa and Con	8 to 92	lagoon
A. eigenmanniorum (14)	14,28% (2)	Brazil	Rio Grande do Sul	Tramandaí river	Laguna dos Patos	Coa	12	lagoon
A. xiru (8)	25% (2)	Brazil	Rio Grande do Sul	Ijuí and Maquiné rivers	Tramandaí-Mampituba, Lower Uruguay	Coa and Con	54 to 127	River, stream
A. cremnobates (10)	40% (4)	Brazil	Rio Grande do Sul	Upper Maquiné rivers	Tramandaí-Mampituba	Coa	870	River

A. douradilho (6)	33,3% (2)	Brazil	Rio Grande do Sul	Lower Maquiné rivers	Tramandaí-Mampituba	Coa	72	River
A. henseli (10)	50% (5)	Brazil	Rio Grande do Sul	Upper Jacuí river	Laguna dos Patos	Coa	272	river

406 **Table 3**. Model selection for the 13 GLMs explaining parasite prevalence. The best-supported models are in bold. AICc (Akaike information criterion); ΔAICc (Delta AICc);
407 Weight (weight of each model in the analysis).

	Parasite frequency models													
Parasite species	**M0**	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**	**M10**	**M11**	**M12**	**M13**
Characithecium triprolatum														
AICc	398.1	5367.6	374.3	374.6	373.7	375.3	4992.4	372.4	387.7	376.9	**366.9**	398.4	388.4	370.5
ΔAICc	31.2	5000.7	7.4	7.7	6.8	8.4	4625.5	5.5	20.8	10.0	**0.0**	31.5	21.5	3.6
Weight	<0.001	<0.001	0.018	0.016	0.025	0.011	<0.001	0.047	<0.001	0.005	**0.750**	<0.001	<0.001	0.125
Characithecium quadratum														
AICc	222.6	1722.7	191.7	188.1	**181.0**	184.0	194.8	182.1	190.6	**179.7**	185.3	197.4	209.2	194.5
ΔAICc	42.9	1543.0	12.0	8.4	**1.4**	4.3	15.1	2.4	10.9	**0.0**	5.6	17.7	29.5	14.8
Weight	<0.001	<0.001	0.001	0.007	**0.252**	0.057	<0.001	0.151	0.002	**0.496**	0.030	<0.001	<0.001	<0.001
Characithecium costaricensis														
AICc	165.7	176.6	165.6	161.0	156.6	160.4	168.3	155.6	160.9	**151.3**	163.3	159.5	165.9	163.3
ΔAICc	14.4	25.3	14.2	9.7	5.3	9.1	17.0	4.2	9.6	**0.0**	12.0	8.2	14.5	11.9
Weight	<0.001	<0.001	<0.001	0.006	0.057	0.008	<0.001	0.097	0.006	**0.804**	0.002	0.013	<0.001	0.002
Characithecium longianchoratum														
AICc	222.6	236.2	219.4	217.3	210.6	213.7	229.7	204.8	221.7	205.9	**197.4**	219.3	221.7	199.7
ΔAICc	25.2	38.8	22.1	20.0	13.3	16.3	32.3	7.4	24.3	8.5	**0.0**	21.9	24.3	2.3

Weight	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.018	<0.001	0.011	**0.739**	<0.001	<0.001	0.231
Characithecium chelatum														
AICc	469.0	370.3	364.9	361.6	**354.2**	358.0	361.9	399.4	425.2	397.4	425.5	427.8	449.5	440.5
ΔAICc	114.8	16.0	10.6	7.4	**0.0**	3.8	7.6	45.2	71.0	43.2	71.3	73.6	95.3	86.3
Weight	<0.001	<0.001	0.004	0.021	**0.831**	0.124	0.018	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Characithecium chascomusensis														
AICc	432.7	382.3	378.5	376.6	**373.4**	**373.4**	379.7	380.8	398.1	383.3	394.9	401.9	410.4	411.4
ΔAICc	59.3	8.9	5.1	3.2	**0.0**	**0.0**	6.4	7.4	24.7	9.9	21.5	28.5	37.0	38.0
Weight	<0.001	0.004	0.032	0.086	**0.424**	**0.419**	0.017	0.010	<0.001	0.002	<0.001	<0.001	<0.001	<0.001
Characithecium robustum														
AICc	153.2	737.2	136.5	134.2	128.3	129.7	150.7	125.4	137.3	128.9	**118.0**	136.5	147.6	129.1
ΔAICc	35.2	619.2	18.5	16.2	10.3	11.6	32.7	7.4	19.3	10.9	**0.0**	18.5	29.6	11.0
Weight	<0.001	<0.001	<0.001	<0.001	0.005	0.002	<0.001	0.023	<0.001	0.004	**0.959**	<0.001	<0.001	0.003

3.3. Ancestral reconstruction of morphological characters

We present in Table 4 a summary of the diagnostic morphological characters proposed for *Characithecium*, which demonstrate the main differences between the species. The ancestral reconstruction for ten discrete characters showed that some of these diagnostic characters are highly variable, being difficult to estimate the most probable ancestral state for the genus. Comparing the AICc values for each models of discrete character transitions (Tab. S4), we can see that the ER model was recovered as the best model for nine of the ten characters analyzed.

Table 4. Summary of characteristics variable within valid species of *Characithecium* based on literature and examined material (Mendoza-Franco et al. 2009; Rossin & Timi, 2015; Gallas *et all.*, 2016). Average size followed by minimum and maximum size in parentheses. μm = micrometers.

Character/species	*C. costaricensis*	*C. chascomusensis*	*C. chelatum*	*C. longianchoratum*	*C. quadratum*	*C. robustum*	*C. triprolatum*
Body length (μm)	280 (215–370)	611 (480–754)	337 (270–426)	450 (351–540)	631 (498–752)	842 (606–1000)	426 (322–555)
Haptor structures							
Ventral anchor length (μm)	34 (33–35)	40 (31–44)	44 (41–46)	56 (51–61)	43 (40–46)	43 (39–48)	37 (32–40)
Dorsal anchor length (μm)	28 (28–29)	36 (27–42)	29 (26–31)	34 (30–38)	34 (32–36)	35 (32–37)	28 (22–35)
Ventral bar shape	straight	U-shaped	V-shaped	straight – slightly U-shaped	straight – slightly U-shaped	V-shaped	straight
Dorsal bar shape	Straight	U-shaped	straight – slightly U-shaped	U-shaped	straight – slightly U-shaped	U-shaped	straight – slightly U-shaped
Medial suture in ventral bar	Absent	present	Present	Present	Present	Present	Absent
Posterormedial projection in ventral bar	Present	absent	Absent	absent	Absent	absent	Present
Reproductive structures							
Rings in the MCO	½ – 1	3 – 4	1 ½	2	2	2 ½	1
Accessory piece shape	Rod-shaped	clamp-shaped	Pincer-shaped	clamp-shaped	Pincer-shaped	Pincer-shaped	Pincer-shaped
Vagina opening direction	Ventral - middle of the body	Marginal-Anterior	Marginal - middle of the body	Ventral - middle of the body	Marginal-Posterior	Ventral - middle of the body	Ventral - middle of the body
Articulation process of accessory piece twisted	Absent	Present	Present	Present	absent	Present	Absent

Regarding characters associated with reproductive organs (MCO, AP, and vaginal opening), we recovered that the ancestor of *Characithecium* possibly already had the base of the MCO articulated to AP, and had AP with clamp-shape or pincer-shape (Fig. 4). Regarding the number of rings in the MCO and the position of the vaginal opening, it was not possible to determine the most ancestral state for the genus. However, it can be expected that the ancestor of *Characithecium* had between two to four rings in the MCO, since this state was recovered as the most likely for the clade composing the majority of the genus species (e.g. *C. costaricensis, C. longianchoratum, C. chelatum, C. chascomusensis*, and *C. robustum*; Fig. 4).

For the characters associated with haptor, the presence of all pairs of hooks with dilated shank and the presence of two hooks with smaller sizes (pairs 1 and 5) were estimated to evolve only once within *Characithecium* and were recovered as most probable ancestral states (Fig. 5). In contrast, regarding the presence of posteromedian projection in ventral bar seems to have evolved independently in *C. costaricensis* and *C. triprolatum* (Fig. 5). However, it was not possible to recover the most likely ancestral state for three characters, such as: (1) size comparison between ventral and dorsal anchors, (2) medial suture in ventral bar, and (3) ventral bar shape (Fig. 5). Despite this, for the ventral bar shape, we can observe the independent evolution of V-shape in two species (e.g. *C. robustum* and *C. chelatum*; Fig. 5).

Figure 4.

Figure 5.

Discussion

Host specialization is often defined by the number of host species used by a parasite, where the greater the number of hosts, more generalist is the parasite species (Poulin et al., 2011). However, phylogenetic distance of the hosts must also be taken into account, where a parasite is considered more specialized when it occurs in phylogenetically closely related hosts (Poulin et al., 2011). For a long time, defining parasitic species between specialist and generalist was the focus within parasitology, where it was believed that this condition was rigid within the evolution of species, in which specialist species lost the ability to colonize new hosts, as they became more and more specialists, and consequently were forced to follow the evolution of their hosts. This led parasitologists to believe that cospeciation was common (known by the term maximum cospeciation) and hosts-shift was very rare (Page, 2003). The idea of maximum cospeciation was so accepted that some

analyzes of reconstructions used the evolution of the parasites to explain the evolution of the hosts (Page, 1995, 2003).

With the present results, we contributed significantly to the update and promote expansion of information regarding the distribution of *Characithecium* species in hosts and geographic areas since the genus was previously reported mainly for a few species of *Astyanax* in Central America, followed by rare records in southeastern South America (São Paulo and Rio Grande do Sul states- Brazil) (Kritsky and Leiby, 1972; Gioia et al., 1988; Mendoza-Franco et al., 2009; Gallas *et al.*, 2016). Here, we observed that *Characithecium* (as a clade) shows a relative high specificity, occurring only in gills of *Oligosarcus* and *Astyanax*, two taxa that are phylogenetically closely related (Mirande, 2018; Wendt et al., 2019). However, within species of *Characithecium*, we observed contrasting levels of specificity, where two species are exclusive to *Oligosarcus* and occur in a few host species of this genus, while others *Characithecium* species are shared with *Astyanax*, having a large number of hosts. However, although these species parasitize a large number of hosts (e.g. 18 species), they use some host species with much higher prevalence than others. Using hosts with low prevalence might be a sign that the parasites are not well adapted to these hosts, instead colonized them by ecological fitting (Agosta et al., 2010, Araujo et al, 2015).

In this sense, many studies have shown that, despite having specificity to one or more hosts, parasites do not lose the capacity to form new associations by ecological fitting, which allows species to access new resources (Janzen, 1985; Agosta et al., 2010). Since then, several studies have shown that host-shifts are common, which may be the result of factors such as phylogenetic and geographic proximity of hosts, as well as biological and ecological conditions (Braga et al., 2015). Braga and colleagues, using monogenoids and Neotropical fishes as models, recovered that the mechanisms that determine parasite sharing vary within each fish group, where for some (*e.g.* Characiformes) the phylogenetic relationship of hosts was the most important factor for carrying out the exchange, while in others (*e.g.* Cichlidae) the geographical distribution had a greater effect. In our data, some species of *Characithecium* had a high share of host species, but these hosts are phylogenetically close related (Wendt et al., 2019). Besides that, previous data demonstrated that *Characithecium* does not occur in other host fish of Characidae, Triportheidae and Bryconidae families, nor in other fish order (Boeger and Vianna, 2006; Cohen et al., 2013), corroborating with the importance of host phylogeny (= close related taxa) as a sharing pattern in Characiformes fishes (Braga et al., 2015).

Regarding the phylogeny of *Characithecium*, our analyses recovered this genus as monophyletic, with high node support. However, the species delimitations based on GMYC and bPTP support a smaller number of entities compared to the morphological delimitation. This result may be mainly a consequence of low genetic variability of ribosomal genes when compared to other genes with higher mutation rates (e.g. mitochondrial genes; Ruttkay et al., 1992; Lemey et al., 2009). In addition, a greater number of species was estimated by bPTP than GMYC, which can be explained by the difference in the species-search methods. While bPTP uses the number of substitutions to model species, GMYC uses time information, which can be influenced by the time calibration (Zhang et al., 2013). Another search found contrasting results for parasite groups within Nematodes, where both models (GMYC and bPTP) recovered a greater number of species when compared to the morphological delimitation (Qing et al., 2019). In this article, the authors used, besides 18S and 28S, fragments of ITS and COI, regions that are known to have greater variability than ribosomal fragments. Besides, the authors carried out a phylogeographic sampling, with many samples analyzed, which may have contributed to the achievement of different putative variability.

Regarding to ancestral state reconstruction of the morphological data, some characters have been shown to evolve independently within the genus, for example, the presence of a posteromedian projection in the ventral bar, which seem to have evolved by convergence in *C. costaricensis* and *C. triprolatum*, and the shape of the ventral bar, which seem to have evolved by convergence in *C. robustum* and *C. chelatum*. In this sense, convergence is a macroevolutionary pattern that has been intensively investigated for many years in several groups of animals (Goswami *et al*., 2011; Burns and Sidlauskas, 2019) and is frequently suggested as a response to the evolution of similar phenotypes in non-sister species, such as presence of wings in bats and birds (Alexander, 2016). This convergent evolution may be the result of similar selective pressures faced by species and imposed by the environment, which develop similar characteristics to live in similar environments.

In the present study, the convergences may be the result of similar selective pressures as demonstrated by the GLM analysis, where for example, high occurrences of *C. costaricensis* and *C. triprolatum* are associated with coastal ecoregions. Since abiotic and biotic variables can regulate species distributions, the GLM analyses has proven to be a great tool to study patterns of parasites diversification, since it makes it possible to test the influence of several variables (alone or together) on the parasitic occurrence

(Huang et al., 2015; Wendt et al., 2018; Bolnick et al., 2019; Mohammed et al., 2020). In the present work, we estimated the influence of a biotic variable (host species) and three abiotic variables (ecoregion, habitat type and altitude class) on the prevalence of *Characithecium* species, in which the seven species demonstrated to have their prevalence influenced in different variables.

Finally, it is important to note that this study was only possible because of the many scientific collections from where the samples were taken. After a careful sampling review of the hosts, they provided us with the study of a large number of host species from different locations, and genetic material to study phylogenetic relationships, which guaranteed an accurate and innovative investigation of evolutionary and ecological aspects of Characithecium in South America. This demonstrates the importance of scientific collections, even when specimens were not collected with these primary objectives (e.g. parasite studies) (Bennett, 2005; Rocha et al., 2014).

Appendix A. Supplementary material

The following are the Supplementary data to this article:

Supplementary 1 - Tables S1–S4

Acknowledgments

This work was funded by CAPES with a doctorate scholarship and was partially funded by Systematics Research Fund 2017/18, both granted to Emilia W. Wendt. Field trips for collecting hosts were partially funded by a CNPq Universal grant (401204/2016-2) to LRM. TPC, during most of the duration of this project, was funded by CAPES with a PNPD postdoctoral fellowship. We also thank to Dr. Vasyl Tkach, Dr. Jason Weckstein, and Dr. Lutz Bachmann for help and comments on the molecular work on parasites, host-parasite relationship and many other discussions.

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CAPÍTULO III

Estimating coevolutionary processes and the effect of opportunity on host-parasite interactions through a multidisciplinary approach

Pretensão de submissão:
Evolution Online ISSN:1558-5646

Estimating coevolutionary processes and the effect of opportunity on host-parasite interactions through a multidisciplinary approach

Emília W. Wendt[1*], Mariana P. Braga[2], Luiz R. Malabarba[1], Walter Boeger[3], Michael Landis[2], Tiago P. Carvalho[1,4]

[1]Laboratório de Ictiologia, Departamento de Zoologia, Universidade Federal do Rio Grande do Sul - UFRGS, Av. Bento Gonçalves, 9500, Bloco 4, prédio 43435, 91501-970 Porto Alegre, RS, Brazil.

[2]Department of Biology, Landis Lab, Rebstock 210, Danforth Campus, Washington University in St. Louis.

[3]Laboratório de Ecologia Molecular e Parasitologia Evolutiva (LEMPE), Departamento de Zoologia, Centro Politécnico, Setor de Ciências Biológicas, Universidade Federal do Paraná (UFPR), Caixa Postal 19073, CEP 81531-980, Curitiba, Paraná, Brazil.

[4]Laboratorio de Ictiología, Unidad de Ecología y Sistemática (UNESIS), Departamento de Biología, Facultad de Ciencias, Pontificia Universidad Javeriana, Carrera 7 N° 43-82, Bogotá D.C., Colombia.

* Corresponding author

Abstract

Host-range expansion is currently accepted as the most plausible null hypothesis to explain the vast majority of host-parasite interactions. This hypothesis is supported by Ecological Fitting that explains how specialist species can turn into generalists and perform host-range expansion without speciation. This information seems to corroborate the current scenario, where new epidemic diseases frequently appear, affecting distinct species of hosts and dispersing widely (e.g. Stockholm paradigm). To examine host-parasite interactions associated with dispersal opportunity, we used a group of monogenoids parasites of freshwater fishes distributed in hydrographic basins in southeastern South America. Monogenoids and freshwater fishes are an excellent system for studying biogeographic and coevolutionary history due to the high specificity of this group of parasites to their hosts, normally occurring in phylogenetically closely related hosts. Besides, the isolation of the hosts in hydrographic basins, which have reticulated histories, resulted in both allopatric speciation and secondary contacts making these freshwater parasites an interesting system to study these complex interactions. From a multidisciplinary study, we recovered the importance of the opportunity for contact

between hosts as a modulator mechanism of the host-parasite interaction. We observe that in links in which hosts have more dispersal opportunity (= coastal basins), the network structure was less specialized than links with few dispersal opportunity (= continental basins). In addition, due to opportunity, global fit methods (PACo) recovered several host-range expansions as the main coevolutionary events that explain the association of *Characithecium* with its hosts (*Oligosarcus* and *Astyanax*). From the ancestral area estimates on the biogeographic analysis, we evaluated two scenarios, one considering the restriction of the occurrence of *Characithecium* to *Oligosarcus* and *Astyanax,* recovering a restricted ancestral area, and another considering that the ancestor of *Characithecium* interacted with other fish species, recovering a wide ancestral area. Scenarios show either a large range extension for *Characithecium* distribution or a widespread ancestral distribution of this genus with some extinction events, being the BAYAREALIKE model the mostly likely to explain area evolution on both scenarios.

Keywords: Network structure, Host-range expansion, Host-parasite evolution, PACo, Parametric Biogeography.

1. Introduction

Host-parasite studies try to understand how organisms and species associations evolved. These studies are highly complex due to several factors, such as the different coevolutionary processes that can shape the evolutionary interactions (e.g. cospeciation, host-range expansion, duplication, or lineage sorting). Additionally, testing for coevolutionary processes requires robust phylogenetic hypotheses with the estimation of divergence times for both the host and the parasite group. Coevolutionary studies have received attention, with the aim of understanding which dynamics influence the evolution of organisms. Therefore, cospeciation and host-range expansion have contrasting effects on the evolutionary patterns observed on hosts and parasites, as discussed below.

The event of cospeciation is the one in which the speciation of one lineage (e.g. host) generates speciation in another (e.g. parasite; Brooks, 1979). This event is thought of as the null model for the coevolution of the host-parasite association due to the high parasitic specificity observed in a large number of species, which occur in one or a few host species (Agosta et al., 2010). Therefore, this model hypothesizes that the degree of parasite specialization is associated with its ability to carry out new host colonization. Consequently, this specialized parasite is obliged to follow the host evolutionary

753 pathway, and cospeciation was accepted as the most plausible process to explain
754 coevolution. Besides, the host-range expansion, or also historically known as "speciation
755 by colonization", assumed that the host exchange was necessarily accompanied by the
756 speciation of the organism, which would make the parasite able to live on this new
757 resource (Ricklefs, 2004).
758 As a way of examining coevolutionary relationships, several methods have
759 emerged, which are based on three main lines of research: (1) analyses based on character
760 optimization and tree reconciliation [e.g. BPA and PACT, (Brooks, 1981; Wojcicki &
761 Brooks, 2005)], (2) event-based methods [e.g. TreeMap and Jane (Page, 1994; Conow et
762 al., 2010)], and (3) global-fit methods [e.g. PARAfit and PACo (Legendre et al., 2002;
763 Balbuena et al., 2013)]. In general, these classes of methods seek to fit the evolutionary
764 history of the parasites over that of the hosts, and therefore, in a more or less profound
765 way, they tend to maximize cospeciation events and minimize host-range expansion.
766 While all these methods assume cospeciation as the null model, they differ in that this
767 process is considered a priori (e.g. event-based methods) or a posteriori (e.g. global-fit
768 methods; Filipiak et al., 2016). This is quite evident in the event-based methods because
769 they assume weights a priori for each coevolutionary event, giving the lowest weights for
770 cospeciation and higher for host-range expansion, and therefore the null hypothesis is
771 difficult to be falsified (Page, 1994; Conow et al., 2010). On the other hand, the global-
772 fit methods provide the opportunity to falsify the null hypothesis (Filipiak et al., 2016),
773 because it is used to quantify the degree of congruence (or incongruence) between two
774 given topologies, and estimate what associations contribute with the congruence of
775 cophylogenetic structure (Balbuena et al., 2013). Cases, where the reconstructed trees are
776 highly congruent, are often interpreted as evidence of cospeciation (Balbuena et al.,
777 2013).
778 Assuming cospeciation as the null hypothesis, we would not be able to explain the
779 occurrence of parasitic species in phylogenetically distant hosts (generalist species),
780 giving rise to the "Parasite Paradox" (Agosta et al., 2010). In this sense, Ecological Fitting
781 (Janzen, 1985) explains how specialist species manage to change hosts without
782 necessarily undergoing speciation (Agosta et al., 2010). That is, even if the parasite
783 specializes in a host, it does not lose the ability to colonize new host species (Araujo et
784 al., 2015). Also, colonizing species can be maintained sub-optimally in some hosts, using
785 them as "stepping-stone" to colonize phylogenetically distant hosts (Araujo et al., 2015).
786 Many other theoretical and empirical studies have shown that host switching is more

common than previously thought and likely more common than cospeciation throughout the diversification of parasitic lineages, even the more specialized ones. In this sense, ecological fitting provides greater compatibility of the parasite to different hosts and guarantees that new colonizations occur if new opportunities arise (Hoberg & Brooks, 2015; Araujo et al., 2015).

These ideas led to the foundation of a new line of research within the coevolution called the Stockholm Paradigm, which suggests the occurrence of host-range expansion as an explanation for the emergence of numerous and new epidemic diseases (Hoberg & Brooks, 2015; Brooks et al., 2019). The Stockholm Paradigm supports the idea that if pathogens manage to disperse and get contact with new hosts (= more opportunity), they will be able to establish themselves and form new populations. This suggests that some species of parasites can manage to have widespread geographical distributions and parasitize several host species once opportunity (e.g. dispersal) is given.

However, host-range expansion makes the system quite complex and often difficult to reconstruct and understand how organisms evolved. Conclusions about coevolutionary events are often difficult to access, mainly if it is generated from a single method, therefore, the use of different methodologies tends to minimize these information gaps. Regarding that, the use of bipartite network analyzes, such as host-parasite interaction, can help us to analyze complex associations and understand how organisms are interacting with each other and what mechanisms can affect the structure of these interactions (Poulin, 2010; Llopis-Belenguer et al., 2020). Also, network analyzes have shown evidence that corroborates the new coevolutionary paradigm and demonstrate that cospeciation is not the most appropriate model for broadly explaining interactions between species (Poulin, 2010; Braga et al., 2018).

In addition to these coevolutionary analyses, biogeographical studies can help us to understand how the distribution of species evolved, what was the role of host dispersal in the evolution of parasites, as well as to estimate which events may be associated with current host-parasite association patterns. Historically, methods for biogeographical analyses have been adapted to coevolutionary analyses, since there is a clear parallel between how organisms disperse and evolve within and between areas and how parasites colonize and evolve with their hosts. Dispersal events carried out by hosts and parasites corroborate the ideas of Ecological Fitting in the sense that by host dispersal, parasites are expanding their opportunity and therefore, phenotypic plasticity is necessary to adapt to new environments (Brooks & Ferrao, 2005).

In this sense, Monogenoidea and freshwater fishes are an excellent system for studying network relationships, biogeography, and coevolutionary history due to the high specificity of this group of parasites (Boeger & Kritsky, 1993). Besides, the isolation of the hosts in hydrographic basins that have reticulated histories (e.g. by river captures) resulted in both allopatric speciation and secondary contacts making these hosts and their freshwater parasites an interesting system to study complex interactions. Monogenoidea is a class of parasitic flatworms mostly primarily parasitizing gills and skin of fishes (Boeger & Kritsky, 1993), which has been subjected to phylogenetic and coevolutionary studies, which documented and discussed cases of cospeciation and host-colonization events (Meinilä et al., 2004; Patella et al., 2017; Benovics et al., 2018; da Graça et al., 2018).

Phylogenetic relationships and interactions of the monogenoid parasites of the genus *Characithecium* and their fish-host species of *Oligosarcus* and *Astyanax* (Characiformes: Characidae), were used as a model for understanding host-parasite relationships associated with ecological preferences and geography of South American freshwaters (Wendt et al., in prep. Cap.2). The host-parasite relationships of these groups are interesting to examine due to different reasons. First, *Oligosarcus* and *Astyanax* are closely related groups (Wendt et al., 2019) and may represent a similar resource for parasites species. Second, the region where these fish species are distributed has a complex biogeographic history (Ribeiro, 2006; Thomaz et al., 2015; Thomaz & Knowles, 2018), which seems to have directly influenced the evolution of these fishes (Wendt et al., 2019). Therefore, due to the specificity of monogenoids, it becomes interesting to examine if these parasites were influenced in the same way as their hosts and contrast different biogeographical areas.

In this sense, we use a multidisciplinary approach to reduce the information gaps on the association of *Characithecium* with their hosts, as well as to evaluate hypothesis of coevolution between these organisms. For this, we use different approaches such as network analysis, coevolutionary methods, and biogeographical models to understand how parasites explore the environment and how it may have influenced their evolutionary history of these species. Also, we evaluated the effect of the opportunity on structuring the host-parasite network and discussed its effect on the evolution of the host-parasite relationships.

2. Material and methods

2.1. Parasite and host sampling, and species distribution

We used the host-parasite interactions sampled by Wendt et al. (in prep, Chapter 2). For parasites, we focused on *Characithecium* (Dactylogyridae), including all seven species (Wendt et al. in prep, Chapter 2). These parasites were collected from different fish hosts, including 17 species of *Oligosarcus* (77% of all *Oligosarcus* species) and 15 *Astyanax* species (close related species to *Oligosarcus* clade, see Wendt et al., 2019). In total, 352 specimens of *Oligosarcus* and 124 specimens of *Astyanax* were sampled from different populations in distinct hydrographic basins of South America (Table S1-S2, Fig. 1).

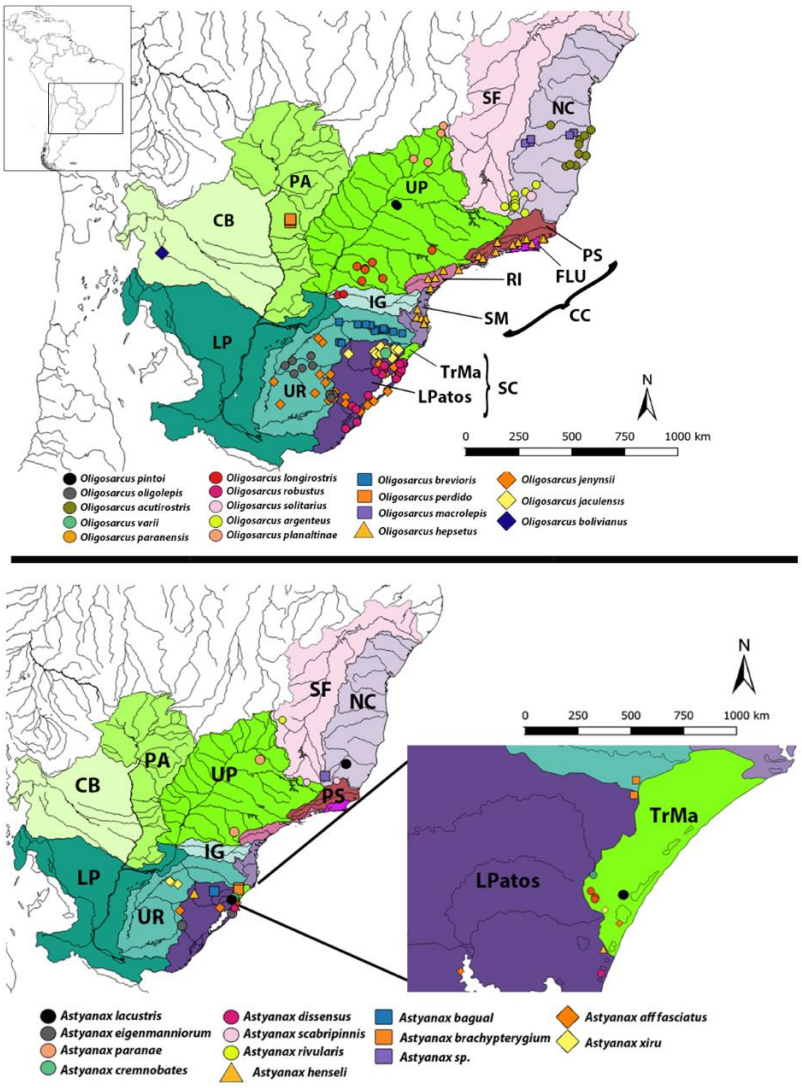

Figure 1. Distribution of *Characithecium* hosts in southeast South America, above: *Oligosarcus* species; below: *Astyanax* species. Maps indicating the areas in southeastern South America (Wendt et al., 2019). Ecoregions (after Abell et al., 2008) abbreviations are: LPatos (Laguna dos Patos) and TrMa (Tramandaí-Mampituba) forming together the SC area (South Coastal in Wendt et al., 2019); SMA (Southeastern Mata

Atlântica), RI (Ribeira de Iguape), FLU (Fluminense) and PS (Paraíba do Sul) forming together the CC area (Central Coastal areas as in Wendt et al., 2019); NMA (Northeastern Mata Atlântica), SF (São Francisco), UP (Upper Paraná), LP (Lower Paraná), IG (Iguaçu), PA (Paraguay), CB (Chaco), UR (Uruguay- with Upper Uruguay and Lower Uruguay as a single area after Wendt et al., 2019).

2.2. Parasite and host phylogeny

For the parasites, we used the time-calibrated tree from Wendt et al. (in prep. Chapter 2), which was estimated using a single nuclear marker (28S), with a fragment of approximately 400 base pairs (bp). We pruned this parasite tree, leaving only the *Characithecium* species and the outgroup *Jainus hexops*. For the host, we used the time-calibrated tree from Wendt et al. (2019), which was estimated using two mitochondrial (COI and ND2), and three nuclear genes (RAG2, Myh6, and S7), with fragments of approximately 714 bp, 1000 bp, 1083 bp, 782 bp, and 743 bp, respectively. The host tree was pruned, leaving only *Oligosarcus* and *Astyanax* species, using the tool "prune clade" in Mesquite Version 3.51 (Maddison and Maddison, 2015). A list of parasite and host species including in this study and the Genbank accession numbers are in Tables S1-S2, respectively.

2.2. Network analysis

2.2.1. Structure of host-parasite Network

To build the host-parasite network, we used the host-parasite associations reported in Wendt et al. (in prep.) (Fig. 2). We organized the interactions data set using two different metrics of parasite occurrence: (1) presence or absence of parasite species in each host species or population (when it was collected from more than one location, Fig. 1), and (2) prevalence of each parasite species in each host species or population. Prevalence is the percentage of parasitized hosts for each species of parasite, in which the number of infected hosts is divided by the total number of hosts examined and multiplied by 100 (Bush et al., 1997).

Then, we used network metrics to observe and describe the patterns of host-parasite interactions, that is, if the host-parasite interactions studied in the present work are specialized and/or nested, and if networks differ whenever examining presence-absence and prevalence interactions. To perform these analyses, we used "networklevel", a function of the bipartite package (Dormann et al., 2008) in the R version 3.6.1 (Core R Team, 2019). We used the Interaction Evenness index (IE) and network-level of

specialization (H2) to detect a pattern of specialization in observed interactions (to both presence/absence and prevalence datasets). The IE index varies from 0 to 1, with 0 being the result of very specialized interactions and 1 being the result of not-specialized interactions. The H2 index ranges between 0 (no specialization) and 1 (complete specialization). For Network Nestedness, we used the overlap and decreasing fill index (NODF), in the binary version for the presence/absence dataset and the weighted version (weighted NODF) for the prevalence dataset. To test the statistical significance of the degree of specialization and nestedness in the networks, we computed these indices for 1000 matrices generated by a null model in which the probability of each interaction is proportional to the number of interactions of the parasite and the host found in the observed matrix, therefore taking into account heterogeneity in host range and parasite richness per host taxon. If the observed patterns in the network structure are significantly different from the null model, these did not emerge from randomness. In order to compare the values of each metric between the different networks, we calculated the Standard scores (Z-score) values, which indicates how much the observed patterns deviate from the null model.

2.2.2. Examining the opportunity for host colonization in the network structure

To test whether opportunity, provided by host dispersal, influenced the structure of the host-parasite networks, we created a model examining the data divided as follows: (1) host-parasite associations in a continental hydrographic basin in southeastern South America; and (2) host-parasite associations in hydrographic basins along the coastal region of southern and eastern Brazil. This idea is based on the assumption that these regions are biogeographically separated (e.g. *Oligosarcus* in Wendt et al., 2019), and dispersal events between these areas are relatively rare. In addition, these two areas (continental and coastal) demonstrated different patterns of dispersion and isolation, which may have influenced differently in host-range expansion and, consequently, the host-parasite network structure. Fishes distributed in the continental basins were isolated at approximately 2 Ma by the Iguaçu and Sete Quedas waterfalls, and these barriers structuring fish species and populations in the La Plata River basin (e.g. Upper Paraná and Iguaçu areas). Approximately 30 years ago, the barrier of the Sete Quedas waterfalls was dismantled after the flooding of the area by hydroelectric construction. Despite this, these two isolations can be translated into less opportunity for contact between species

and may generate a higher parasite specificity of occurrence in some hosts. On the other hand, species with a coastal distribution were able to disperse within that area after successive transgressions and regressions of sea level during the Pleistocene (Thomaz & Knowles, 2018; Wendt et al., 2019). These successive episodes of dispersion can be translated into a higher opportunity for contact between hosts, which may have influenced the host-range expansion for parasites in these fish and, therefore, result in a different network structure.

Therefore, we hypothesize that host-parasite associations in continental areas are more specialized and less nested, and host-parasite associations in coastal areas are less specialized and more nested, due to less and greater opportunity for contact between hosts, respectively. We tested this hypothesis using the metrics and null model described above. Here, if the observed patterns in the network structure are significantly different from what is generated by the null model, these patterns did not emerge simply from a random process but opportunity.

2.3. Inferring processes of coevolutionary diversification

We used the Procrustes Approach to Cophylogeny (PACo) to estimate coevolutionary processes and to observe the patterns of host-parasite associations. We performed this analysis for three different datasets: all host-parasite links, only coastal, and only continental links. We performed these analyses with the same objective proposed in item 2.2.2, where we seek to estimate the effect of the opportunity on the host-parasite interactions. PACo is a global fit method for cophylogenetic analysis based on Procrustes analysis (Balbuena et al., 2013), where we can examine congruence (or incongruence) between two given topologies of phylogenetic relationships (e.g. host and parasite) and identify the contribution of each host-parasite link to the cophylogenetic congruence. The goodness-of-fit statistic (m^2_{XY}) inform the degree of congruence, where this value is inversely proportional to the topological congruence, that is, the higher is the m^2_{XY}, the smaller is the congruence between the two trees. In this case, high congruence can be interpreted as cospeciation events that occurred between host and parasite evolution, and low congruence indicates host-range expansion (Balbuena et al., 2013). PACo uses the phylogenies of hosts and parasites, collecting the phylogenetic distance information between terminals of each tree. It is necessary to include a host-parasite association matrix, where 0 corresponds to absence, and 1 corresponds to the presence of parasite species in a host species. Then, PACo searches for the best global-fit of host-

parasite association, performing statistical analyzes where a randomization procedure can establish significance. Also, this method tests the importance of each host-parasite link in the global-fit through of squared residual analysis, which, together with their 95% confidence intervals, are estimated using a jackknife method (Balbuena et al., 2013).

The PACo accepts multiple interactions between species, where parasite species can occur in more than one host species and host species having more than one parasite species. Also, the method does not require a priori determinations to events and does not determine which events generated the congruence or incongruity between the trees. In other words, PACo performs a residual analysis and uses this data to generate interaction patterns, where links with equal and low residuals (below the median residual value) have the same interaction pattern and high chances of having evolved from the same event (such as host-range expansion, extinction, and duplication). Therefore, it is worth saying that PACo performs an estimate of patterns of interaction between host-parasite, where low residues are indicative of coevolution events.

Figure 2.

2.4. Ancestral range estimation of geographic areas and host-parasite associations

We use event-based analyses to evaluate biogeographical processes and host-parasite association delineating *Characithecium* species distribution. These methods estimate cases of allopatric speciation (vicariance), allopatric with secondary contact (dispersal), and sympatric speciation (within-area speciation). Firstly, we estimate the ancestral area range for fish hosts to use the results of this analysis to constrain the parasite ancestral area range. So, we constructed the same taxon-area matrix for *Characithecium* and fish species distributions using geographic operational units. The geographic unit delimitation is similar to the Freshwater Ecoregions of the World (FEOW) proposed by Abell et al. (2008). However, some of the proposed Freshwater Ecoregions were joined to reduce the number of areas and parameter space: (1) Chaco (CB), Paraguay (PA) and Lower Paraná FEOW's were joined as the Lower La Plata (LP); (2) Lower and Upper Uruguay FEOW's were joined as Uruguay (UR); (3) Laguna dos Patos (LPatos) and Tramandaí-Mampituba (TrMa) FEOW's were joined in South Coastal (SC); (4) Southeastern Mata Atlântica (SMA), Ribeira de Iguape (RI), Fluminense (FLU) and Paraíba do Sul (PS) FEOW's were joined in Central Coastal (CC). Therefore, a total of eight geographical units were used in southeastern South America (Fig. 1), where five are located in continental areas such as São Francisco (SF), La Plata (LP), Upper Paraná (UP), Iguaçu (IG), and Uruguay (UR); and three coastal drainage areas: North Coastal

(NC; corresponding to Northeastern Mata Atlântica ecoregion of Abell et al., 2008), Central Coastal (CC), and South Coastal (SC) (Fig. 1). The presence/absence of parasite and host species within the operational geographic units were coded based on distributional data of *Characithecium* species (Wendt et al., 2020; in prep.) (Table S3 to S5).

We perform an ancestral range estimation to host species of the clade containing *Oligosarcus* and *Astyanax* species. We used geographic information to restrict and allow dispersal: the connection between coastal drainages during Pleistocene (2.8 Ma) and the isolation of the Iguaçu and Upper Paraná basins (2.0 Ma) of the other drainages of La Plata (continental region) through the formation of Iguaçu and Sete-Quedas waterfalls in the late Pleistocene (Stevaux, 1994; Miller et al., 2011; see also Landscape Evolution Models in Wendt et al., 2019). In the package BioGeoBEARS, this geographic information was used to construct dispersal multipliers matrices, changing the dispersal rates to correspond the connection and isolation of each geographical event into four distinct time frames: 1) 20-5 Ma, 2) 5-2.8 Ma, 3) 2.8-2.0 Ma and 4) 2.0-present. The oldest time considered in the geographic analysis was based on the estimated maximum age for the clade composed by *Oligosarcus* and *Astyanax* (recovered with divergence time estimation using fossil data as calibration points), followed by the estimated time for *Oligosarcus* clade (~5 Ma), then by the beginning of the Pleistocene (2.8 Ma), and the estimated age of formation of the Iguaçu and Sete-Quedas waterfalls (2.0 Ma). The maximum number of areas occupied by a lineage was set to eight, which is the maximum number of areas currently observed in any of the fish species analyzed (e.g. *A. lacustris* occurs in eight areas).

After this, we evaluated the ancestral range evolution for parasites using two distinct approaches. First, we performed the analysis without including any prior information on dispersal rates or areas permitted. Then, we performed the analysis using the information on the ancestral range estimates of the host lineages, in which we inform the likely areas occupied in the ancestral state of the hosts. For example, if it has been estimated that the fish occurred in an area corresponding to SC, UR, and LP in 20 Ma ago, we then allow these same areas of occurrence for the parasites in the same period. This is based on the parasite's dependence by the host to be able to disperse. Therefore, the area occupied by the host species can also be occupied by the parasite species. So, we considered three different times for parasite ancestral range: 1) 20-10 Ma, 2) 10-2.8 Ma, 3) 2.8-present.

We examine both host and parasite ancestral range using model-based analytical methods in historical biogeography using the package BioGeoBEARS in R (Matzke, 2013, 2014). For hosts, we evaluate only models that accommodate vicariance (e.g. DIVALIKE, DEC, respectively Ronquist, 1997; Ree and Smith, 2008), since these models include most biogeographical events associated with the diversification of fish across drainage basins of southeastern South America (e.g. Menezes, 1988; Ribeiro, 2006; Machado et al., 2018). For parasites, we evaluate these same models (e.g. DEC and DIVALIKE) but also BAYAREALIKE that do not include vicariance as a possible cladogenetic event (Landis et al., 2013; Matzke, 2014).

3. Results

3.1. Network analysis

3.1.1. Structure of host-parasite network

We present the host-parasite network, using the presence/absence and the parasite prevalence, where each host-parasite interaction is represented by black cells (or grey cells in the case of prevalence; Fig. 3). We note that the network structure is different, depending on what data we use (occupancy or prevalence), presenting different values of z-score and p-value (Table 2). When we analyze the structure of the network using the presence-absence information (occupancy), the network is nested (high value of NODF) and not specialized (high value of IE and low value of H2; Table 2). In contrast, analyzing the network structure using prevalence data, the network is less nested (low value of weight NODF), and is very specialized (low value of IE and high value of H2, Table 2). Due to this difference in the structure of the host-parasite network using the prevalence data, the opportunity influence analyzes on different regions (see below) were performed only with this dataset.

Figure 3.

3.1.2. Examining the host opportunity in the network structure

The structure of the host-parasite network demonstrated significant differences between the tests when we examine coastal and continental interactions separately (Table 2). In continental ecoregions, where there has been less opportunity for parasite to colonize new hosts, the host-parasite network is less even and more specialized (Table 2), where parasite species show higher prevalence in some hosts than in others (Figure 4). The network including only host-parasite interactions in coastal ecoregions, which are associated with more dispersal opportunity, is more even and less specialized (Figure 4).

This means that parasite species have a similar distribution in the different fish species, showing similar prevalence values.

Figure 4.

Table 2. Network analysis in three different scenarios (all host-parasite links, only continental host species links and only coastal host species links). The significance of the analyzes is based on the p-value and z-score value. Prev. = prevalence interaction. H2 = network-level measure of specialization.

	All links		**Continental links**	**Coastal links**
Metrics	**Presence/ Absence**	**Prev.**	**Prev.**	**Prev.**
NODF or weighted NODF	46.215	13.731	16.280	12.767
p-value	<0.0001	0.962	0.996	0.999
z-score	4.677	-1.799	-2.824	-4.068
Interaction evenness	0.805	0.763	0.703	0.750
p-value	<0.0001	0.999	0.999	0.999
z-score	5.491	-315.98	-195.55	-224.785
H2	0.000	0.542	0.549	0.531
p-value	0.999	<0.0001	<0.0001	<0.0001
z-score	-5.491	315.98	195.55	224.785

3.2. Inferring processes of coevolutionary diversification

The global-fit analysis using PACo, performed with all host-parasite links (Fig. 5), recovered partial congruence between host and parasite phylogenies (m^2_{XY} = 14316.28; *p-value* = 0.02). On the other hand, the data using only continental or coastal links (Fig. 6) did not recover any significative congruence between the phylogenies. However, the global-fit analysis recovered higher incongruence (m^2_{XY} value) within coastal links (m^2_{XY} = 11953.44; p-value = 0.21) than when compared to continental links (m^2_{XY} = 3385.05; *p-value* = 0.22).

In analyses using all host-parasite links, the residual values together with the divergence time estimates to parasite and host species (indicated by the time scales at oldest node of *Characithecium* and *Oligosarcus/Astyanax*), suggests evolutionary connection between groups. The congruence (all links analysis) recovered by PACo is mostly supported by *C. costaricensis* and the clade composed by some *Astyanax* species (Figure 5), for example, *A. brachypterigyum* and *A. rivularis*, distributed in south and north of Brazil, respectively. This suggests that the ancestor of *C. costaricensis* (Type - Figure 5), which diverged approximately 8 Ma ago, was associated with the ancestor of the *Astyanax* Clade 3 in the Figure 2, and other associations represent later colonizations in more recent time.

When we observed the squared residual for analysis with all links (Fig. 5), associations of *Characithecium* species with *Oligosarcus* species have smaller residues (below residual average) than compared with associations with *Astyanax* species (Figure 5). This demonstrates that *Characithecium* species have a stronger relationship and more interactions with *Oligosarcus* than with *Astyanax*, with most associations with *Astyanax* being probably acquired through host-range expansion from associations with *Oligosarcus* or result of repeated extinction events of parasites.

In both *C. robustum* (P1- Figs. 5-6) and *C. longianchoratum* (P5), which are associated only with *Oligosarcus* species, we observed different interaction patterns of these parasites in continental and coastal links. *Characithecium robustum* shown more links with the continental hosts than coastal hosts, and this links presented less squared residues in *O. jenynsii* and *O. longirostris* species. On the other hand, *C. longianchoratum* presented the same number of continental and coastal links, but smaller residues in continental associations (Fig. 6). For *C. chascomusensis* (P2), the continental links presented smaller residues and were formed only by interactions with *Oligosarcus* species, in contrast to the coastal links where the residues were larger and more variable, including a larger number of hosts species (Fig. 6).

For *C. triprolatum* and *C. quadratum* (P3 and P6, respectively), which would have recently diverged at approximately ~ 3 Ma (Figure 2), the similar residue values (Fig. 5) and the presence of both species of parasites in the clade composed by *O. robustus*, *O. oligolepis* and *O. hepsetus* suggests that the ancestor of these parasites colonized the ancestral lineage of these species. Therefore, *C. triprolatum* and *C. quadratum* would come from a duplication event within the coastal clade composed by *O. robustus*, *O. oligolepis*, *O. hepsetus* and *O. acutirostris*. After that, *C. robustum* appears to be restricted to *Oligosarcus* species, unlike *C. triprolatum*, which colonized many other species of *Oligosarcus* and *Astyanax* (Figure 2 and 5). Then, *C. chelatum* (P4), which would have diverged to approximately 4 Ma, presented smaller residues for the associations with the continental clade formed by the species *O. pintoi*, *O. paranensis* and *O. planaltinae*, which indicates a stronger interaction with these hosts and the other associations resulting from later colonization (Figs 5-6).

Figure 5.

Figure 6.

3.3. Ancestral range estimation

For ancestral range estimation to host species (*Oligosarcus* and *Astyanax*), we recovered that DEC as a better fitting model of area evolution than DIVALIKE (Table 3). In this analysis, the South Coastal area was recovered as an ancestral area (Fig. 7). The ancestral lineage of *Oligosarcus* would have expanded its distribution in the period between 10 to 5 Ma, dispersing to areas such as Uruguay, Lower La Plata, Iguaçu, Upper Paraná, and North Coastal (Fig. 7). After that, at approximately 5 Ma, *Oligosarcus* went through vicariance, where one clade was restricted to the continental region and another to the coastal region.

Contrasting ancestors to modern species of *Astyanax,* in general, began its range expansion later (~ 5 Ma), where some species dispersed to others coastal region (e.g. *A. rivularis*, *A. scabripinnis*) and others lineages remained restricted to South Coastal area (e.g. *A. douradilho*, *A. bagual*) or dispersed throughout the continental region (*e.g. A. paranae*, *A. eigenmanniorum*). For both *Oligosarcus* and *Astyanax*, the secondary contact of some lineages after 2.8 Ma was evidenced, connecting coastal drainages with continental drainages (e.g. South Coastal with Uruguay, and North Coastal with São Francisco).

For the ancestral range estimation of parasites, we recovered the BAYAREALIKE as the best-fit model for both analyses: using a host-distribution constraining approach on dispersal and area allowance and without these constraints. These different approaches, however, recovered different ancestral area estimates, as well as different likelihoods and parameter values (extinction and dispersal; Table 3, Figs. 8). Based on these values, we observed that the BAYAREALIKE recovered for data without host constrain showed a higher likelihood and lower values of dispersion and extinction when compared to the BAYAREALIKE with host constrain, indicating this as a better model to estimate the ancestral area of the parasites (Table 3). In addition, the BAYAREALIKE values for the data with host constrains showed very similar values of likelihood and AICc when compared with DEC and DIVALIKE, indicating that these three models should be equally considered (Table 3).

In estimating the ancestral area without taking into account the ancestral distribution of hosts, the BAYAREALIKE model recovered a large ancestral area for *Characithecium* formed by Upper Paraná, Iguaçu, Lower La Plata, Uruguay, South Coastal, Central Coastal and North Coastal (Fig. 8). Contrarily, when we used the host information for examining area evolution on parasites, we recovered a restricted ancestral

area that corresponds to South Coastal (SC) and then following distributional expansion, which indicates several dispersal events in *Characithecium* (Fig. 8). The ancestral area recovered for the *C. quadratum* + *C. triprolatum* clade corresponds to a large range expansion processes expanding to Central Coastal, Uruguay, and Lower La Plata, and after sympatric speciation within this area. Later, *C. triprolatum* dispersed to other areas such as Upper Paraná, North Coastal, and São Francisco.

The BAYAREALIKE model recovered a northward distributional expansion for *C. costaricensis* at around 10Ma from South Coastal to Uruguay, and for all other areas of the coastal region (Central Coastal, North Coastal, and São Francisco). For *C. longianchoratum*, the distribution expansion would have occurred from South Coastal to Uruguay, Lower La Plata, Upper Paraná, and Central Coastal to approximately ~ 5 Ma, and for *C. chelatum* the area expansion would have occurred ~ 4 Ma ago, reaching a wide distribution by continental and coastal basins. Another sympatric speciation was estimated in the Iguaçu, Lower La Plata, Uruguay and South Coastal areas, around ~ 3 Ma ago, where the species *C. robustum* remained restricted to that area and the species *C. chascomusensis* managed to disperse and expand its area to include Upper Paraná, Iguaçu, North Coastal, and Central Coastal (Fig. 8).

Figure 7.

Figure 8.

Table 3. Comparison of the different models (DEC, DIVALIKE and BAYAREALIKE) of ancestral range estimation to parasite species (*Characithecium*) and to host species (*Oligosarcus* and *Astyanax*), based in the present ecoregion distribution. In bold are the best models explaining the area evolution to each group (parasite and host). # number of parameter estimates; AICc= Akaike Information Criterion; AICc weights= AICc weighted; ΔAIC= delta AIC.

Models	**Parameter estimates**				**Likelihood-ratio test**	**Information criteria**		
	Ln *L*	#	*d*	*e*	*P*-value	AICc	AICc weights	ΔAICc
	Parasite ancestral range estimation - Without host constrains							
DEC	-30.79	2	0.47	0.19	<0.0001	68.58	0.20	1.7
DIVALIKE	-30.31	2	3.54	3.66	<0.0001	67.62	0.33	0.74
BAYAREALIKE	-29.94	2	3.51	3.63	<0.0001	66.88	0.47	0.0
	Parasite ancestral range estimation - With host constrains							
DEC	-38.37	2	0.28	0.13	<0.0001	83.75	0.14	3.42
DIVALIKE	-38.97	2	0.26	0.14	<0.0001	84.94	0.078	4.61
BAYAREALIKE	-36.66	2	0.083	0.071	<0.0001	80.33	0.78	0.0
	Host ancestral range estimation							
DEC	-123.7	2	0.13	1.0e-12	<0.0001	251.9	0.99	10.3
DIVALIKE	-128.9	2	0.16	1.0e-12	<0.0001	262.2	0.0059	0.0

4. Discussion

4.1. Network analysis

The use of network theory in studies of host-parasite interactions contributed to our understanding of host-parasite evolution (Poulin, 2010; Braga et al., 2018; Llopis-Belenguer et al., 2020). Network structure can show us how one species is interacting with the others and present us with a more detailed view of the system (Poulin, 2010), and together with phylogenetic information, can help us to understand how species coevolved over time. Therefore, it is interesting to understand which mechanisms are associated with different structures recovered by network analyses.

Here, we observed that the structure of the host-parasite network is different depending on what type of interaction data. When we consider only the presence or absence of the host-parasite interaction, we observe a significantly nested (high value of NODF) and non-specialized network (high value of IE and low value of H2). In other words, this network has some interactions forming a subset of other interactions, and the parasite species are distributed equally among all hosts, without specialization for any of them. In contrast, the network structure presented by the interaction with prevalence data, which can also be called "strength of interaction", the network was found to be non-nested and very specialized, informing us that the parasite species interact differently with the hosts, occurring preferentially in some species and rare in others.

In this sense, parasites tend to have aggregate distribution, which few hosts will have most of the parasite species, reflecting in the different prevalence values in each interaction pair (Shaw & Dobson, 1995). However, two main mechanisms can be associated with the success of the host-parasite interaction. One of them is known as compatibility (=capacity of colonization), which is estimated that intrinsic characteristics of the parasite could explain the patterns of interactions between host and parasite (Combes, 2001; Bandilla et al., 2005; Poulin, 2013; Wendt et al., 2018). However, more recent studies propose that opportunity might be more important than compatibility during the colonization of a new host because of Ecological Fitting, for instance, when parasites have phenotypic plasticity to guarantee new colonization without new adaptations (and subsequent speciation) being necessary for this (Agosta et al., 2010). In addition, parasites can remain sub-optimal in some hosts until reaching the "best" host (Araujo et al., 2015). Therefore, currently, the host-parasite interaction, as well as the different structures in the network, seem to be more influenced by the presence of

ecological opportunity between different hosts than with capacity (Araujo et al., 2015; D'Bastiani et al., 2020).

In our work, the host dispersal opportunity seems to be a determining factor for the structuring of the host-parasite networks studied here, in which we compared links occurring in continental and coastal basins in the southeastern region of South America. We observed that host-parasite associations in coastal basins had shown a pattern of interaction without parasitic specialization, in contrast to associations in continental basins, which were more specialized. We suggest that these different network structures between *Characithecium* and their hosts are linked to the evolutionary history presented by the fish, in which well-structured clades evolved separately in the continental and coastal regions (Wendt et al., 2019). We suggest that the low parasitic specialization in the coastal region was the result of more frequent host-range expansion due to the opportunity for contact between the fish of that region at different times in the past, as estimated for *Oligosarcus* (Wendt et al., 2019). This region is known for its high evolutionary complexity, where it is estimated that several fish species had its evolution influenced by sea-level fluctuation during the Pleistocene (Thomaz et al., 2015; Tschá et al., 2017; Wendt et al., 2019). With this fluctuation, currently isolated hydrographic basins were connected, providing dispersion and contact routes for the fish (Thomaz & Knowles, 2018), in which they also allowed the parasites to make frequent hosts-switching.

4.2. Coevolutionary and biogeographic diversification

Our hypothesis suggests that the opportunity acted as the primary modulating mechanism of the association between *Characithecium* and its hosts are also evident when we evaluate the results generated by the analyzes of coevolution and biogeography. Although three species of *Characithecium* showed high evolutionary complexity, occurring in a large number of hosts, we can obtain usefull information about the probable evolutionary history associated with these parasites. The analysis of the PACo recovered little congruence between the topologies of the hosts and parasites, showing that the speciation of the parasites was little influenced by the speciation of the hosts. Since the congruence quantifies what node in a given tree-map corresponds to the same position in the other tree (Balbuena et al., 2013), the difference in the number of hosts and parasites species sampled here was already indicative of low congruence, indicating that the parasites have speciated at a slower rate than the hosts. Furthermore, knowing that

congruence is very rare in nature and that high congruence can be interpreted as evidence for cospeciation (Balbuena et al., 2013), other coevolutionary processes are necessary to explain the association between *Characithecium* and its hosts.

In this sense, the residual values in the coevolutionary analysis (PACo), together with the divergence time to hosts and parasites, allowed us to infer a possible evolutionary history associated with the interaction of each species of *Characithecium* with its hosts. A plausible coevolutionary scenario, which would better explain the association between *Characithecium*, *Oligosarcus*, and *Astyanax*, indicates a large number of host-range expansion. These results would be challenging to explain if we accepted the hypothesis of maximum cospeciation. However, they fit in the current coevolutionary hypotheses, where the opportunity for contact between two hosts is the determining factor for the success of new colonization (Agosta et al., 2010; Araujo et al., 2015; Hoberg & Brooks, 2015).

Thus, the sharing of monogenoid species among fish of the order Characiformes proved to be more influenced by the geographic distribution of host than by host phylogeny (Braga et al., 2015), so if these fishes had overlapping geographical ranges, their parasites would likely perform host-range expansion. In our work, the phylogenetic proximity of the fish added to their reticulated evolutionary history within the southeastern region of South America (Wendt et al., 2019), seems to have facilitated host-range expansion, with this event appearing to have occurred several times within *Oligosarcus* and between *Oligosarcus* and *Astyanax*. This allowed the species of *Characithecium* to colonize a large number of hosts, as well as to reach wide geographical distribution, more so on coastal areas than in continental areas.

In this sense, when we link the information from the coevolutionary and biogeographic analyzes, it is evident the high capacity of *Characithecium* to colonize new host resources. However, two evolutionary scenarios are considered for the evolution of *Characithecium*. One of these scenarios suggest that the expansion of the area of the parasites occurred together with the expansion of the area of the fish species analyzed here. The other scenario suggests that the ancestor of *Characithecium* probably had an association with other fishes and occurred in a very wide ancestral area, colonizing and later specializing in *Oligosarcus* and *Astyanax*.

An alternative to identify and distinguish a probable evolutionary scenario would be the use of more variable markers, which would provide us with more detailed information on the genetic variability of the distinct populations of *Characithecium*

species in the different host species. For example, with a phylogeographic analysis, it would be possible to estimate patterns of diversification between different populations (Avise, 2000; Fraija-Fernández et al., 2017; Bueno-Silva & Boeger, 2019), and perhaps it could contribute to the study of different species of *Characithecium* and thereby evaluate which populations are closest phylogeographically, being able to distinguish between distinct biogeographic scenarios.

The analyses employed here allowed us to observe the large dispersal capacity of *Characithecium* species. This suggests that these parasites have a large fitness space (sensu Agosta & Klemens, 2008) and tend to "occupy" different species of fish to disperse. Based on the ecological fitting, parasites can perform host-range expansion without evolving new host utilization capability and then perform large dispersions (Brooks & Ferrao, 2005). Also, based on the analyses of BAYAREALIKE without host constrain, the data suggest that *Characithecium* probably had a wide dispersion in the southeastern region of South America, having initially colonized *Astyanax* species, and later colonized and specialized in *Oligosarcus*. When we observe the dispersal and extinction parameters estimated by the analysis with host constrain, we observed that this model was not satisfactory to explain the area evolution of *Characithecium*. This may be associated with uncertainty regarding the most likely ancestral area for *Oligosarcus* and *Astyanax*, and we suggest may be solved in future increasing the sampling effort within the largely geographically distributed genus *Astyanax*.

Appendix A. Supplementary material

The following are the Supplementary data to this article:

Supplementary 1 - Tables S1–S6

Acknowledgments

This work was funded by CAPES with a doctorate scholarship and was partially funded by Systematics Research Fund 2017/18, both granted to Emilia W. Wendt. Field trips for collecting hosts were partially funded by a CNPq Universal grant (401204/2016-2) to LRM. TPC, during most of the duration of this project, was funded by CAPES with a PNPD postdoctoral fellowship.

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Conclusões Gerais

A presente tese utilizou abordagens multidisciplinares com o objetivo de reconstruir hipóteses filogenéticas para *Oligosarcus* e seus parasitos de brânquia, *Characithecium*, e estimar a provável história evolutiva desses organismos.

Para tal, primeiramente, o **Capítulo I** focou nas relações filogenéticas dos hospedeiros, realizando uma estimativa de tempo de divergência para *Oligosarcus* e *Astyanax*, bem como uma reconstrução ancestral de área. Nesse estudo, *Oligosarcus* foi recuperado como monofilético, com um alto suporte filogenético, e divergindo a aproximadamente 5 milhões de anos atrás (Ma). Dentro de *Oligosarcus*, foram recuperados dois clados com semelhante riqueza de espécies e tempo de divergência (~3 Ma), e também com com alto suporte filogenético. A reconstrução ancestral de área recuperou que o ancestral de *Oligosarcus* provavelmente ocorria em uma ampla área geográfica do sudeste da América do Sul, composta pelas ecorregiões do Alto Paraná, região costeira Norte e região costeria Central. Em seguida, um evento de vicariância foi estimado para o inicio da radição do gênero, permanecendo uma linhagem restrita à região continental (Grupo Continental) e outra restrita à região costeira (Grupo Costeiro).

Para o Grupo Costeiro, o qual se dispersou de norte a sul da região costeira no sudeste da América do Sul, foi recuperada a importância dos eventos de transgressão e regressão do nível do mar, durante o Pleistoceno (~2.8 Ma), para dispersão e diversificação desse grupo. Por outro lado, para o Grupo Continental, o qual se dispersou pelas bacias do Alto e Baixo Paraná, Paraguai, Iguaçu, Chaco e Uruguai, foi recuperada a influência das cataratas do Iguaçu e Sete Quedas como evento de diversificação desse clado.

Em seguida, foi realizado no **Capítulo II** uma extensa investigação sobre a diversidade parasitária em brânquias de 17 espécies de *Oligosarcus*, bem como de 15 espécies de *Astyanax*, as quais são filogeneticamente próximas à *Oligosarcus* e com ocorrência simpátrica à alguma espécie desse gênero. Foram identificadas 7 espécies de *Characithecium*, um grupo de parasitos específico de brânquias de *Oligosarcus* e *Astyanax*. Posteriormente, através de análises moleculares, estimou-se a relação filogenética entre esses parasitos, recuperando a monofilia dessas sete espécies.

Além disso, foram investigadas se algumas características ecológicas estariam associadas a diferentes taxas de prevalências observadas para cada espécie de parasito nos seus respectivos hospedeiros. Para tal, foram testadas quatro variáveis, sendo elas:

espécie hospedeira, ecorregião, altitude e tipo de hábitat (lagoa, rio ou riacho). Observou, no geral, que cada espécie de *Characithecium* possuiu variáveis diferentes associadas ás suas taxas de prevalência, sendo algumas delas influenciadas pelas espécies de hospedeiros e outras influenciadas pela combinação entre ecoregiões e tipo de hábitat. Por fim, esse capítulo também abordou uma análise de reconstrução de estado ancestral para 8 caracteres morfológicos, sendo observado a evolução convergende de alguns deles.

Após possuir o conhecimento sobre as relações filogenéticas dos peixes (hospedeiros) e dos parasitos, e de identificar as associações entre esses indivíduos, essa tese finaliza com um estudo detalhado sobre a estrutura das interações entre parasitos e hospedeiros e a história coevolutiva dessas associações, bem como realiza uma estimativa de área ancestral para ambos os táxons. Apartir de análises de network, o **Capítulo III** apresenta como esses indivíduos interagem e quais mecanismos parecem influenciar na estrutura da rede. Para isso, a rede de interações foi analizada utilizando dados de presença/ausência e dados de prevalência. Os resultados dessas análises demonstraram diferença significativa na estrutura dessas redes, onde os dados de prevalência demosntraram que os parasitos interagem de forma diferente entre os hospedeiros. Ou seja, apesar de muitas espécies de parasitos ocorrerem em diversos hospedeiros, muitas relações possuem baixas prevalências e apenas um ou poucos hospedeiros possuem altas prevalências.

Além disso, esse capítulo abordou o papel da oportunidade de dispersão (dos hospedeiros) como mecanismo modulador da estruturação das redes, influenciando a história coevolutiva desses indivíduos. Para tal, separamos as associações em dois grupos, um deles sendo composto por interações entre hospedeiros e parasitos presentes em drenagens continentais, as quais tiveram menos oportunidades de dispersão, e outro grupo composto por interações em drenagens costeiras, o qual teria realizado sucessivos episódios de dispersões. Com isso, foi recuperado que hospedeiros que tiveram mais oportunidades de dispersão parecem ter proporcionado aos parasitos uma maior oportunidade de transmissão lateral, sendo recuperadas redes menos especializada nas regiões costeiras do que nas regiões continentais.

As análises coevolutivas, realizadas a partir da utilização de PACo demonstrou uma história evolutiva de *Characithecium* mais ligada a espécies de *Oligosarcus* do que a espécies de *Astyanax*, devido aos baixos valores de resíduos nas interações dentro desse gênero.

Por fim, análises de reconstrução ancestral foram recuperadas usando dois cenários evolutivos para os parasitos. Em um deles utilizamos a informação de área ancestral dos hospedeiros (*Oligosarcus* e *Astyanax*) para restringir a área ancestral dos parasitos. Esse cenário recuperou a região costeira sul como área ancestral para *Characithecium*, e diversas dispersões posteriores a partir de 10 Ma. Por outro lado, um outro cenário, o qual foi realizado sem informações a priori sobre a distribuição dos hospedeiros, recuperou uma ampla área ancestral para *Characithecium*, indicando que esses parasitos provavelmente eram associados a outras espécies de peixes no início de sua radiação, as quais possuíam ampla dispersão. Nesse cenário, a associação com *Oligosarcus* e *Astyanax* teria ocorrido posteriormente a partir de novas colonizações e consequente extinção nos hospedeiros ancestrais.