UFRGS

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ASPECTOS EVOLUTIVOS EM STEVARDIINAE (OSTARIOPHYSI: CHARACIDAE): FILOGEOGRAFIA E FILOGENIA

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
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Aprovada em _____de_____de 2015.

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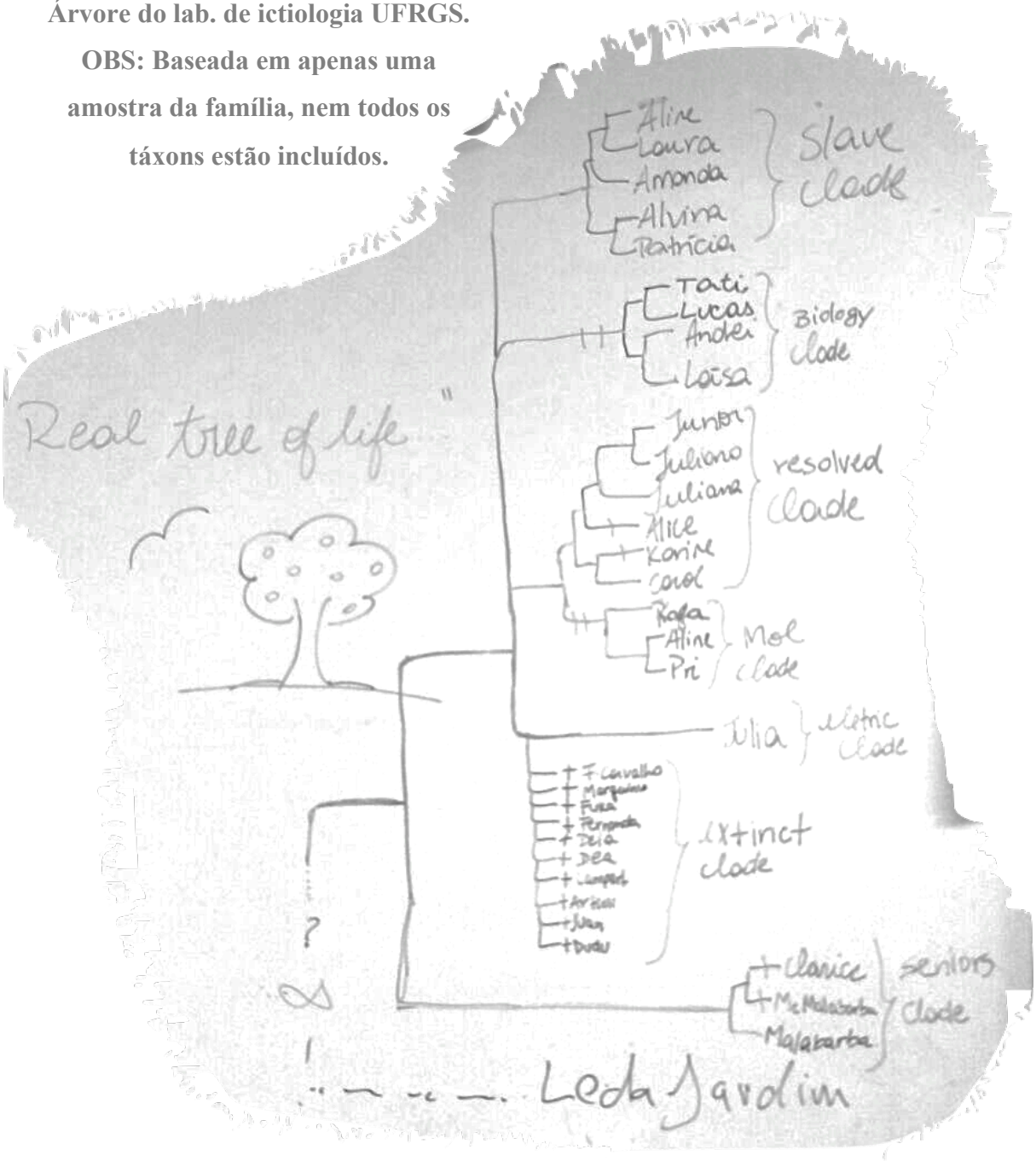
OBS: Baseada em apenas uma amostra da família, nem todos os táxons estão incluídos.



"Infinitas formas, as mais belas e mais maravilhosas, evoluíram e continuam evoluindo."

Charles Darwin

“There are many hypotheses in science that are wrong. That's perfectly alright; it's the aperture to finding out what's right. Science is a self-correcting process. To be accepted, new ideas must survive the most rigorous standards of evidence and scrutiny.”

Carl Sagan

Agradecimentos

"Life's biggest battles often are fought alone"
Breaking all illusions (Dream Theater)

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Mother
I am always close to you
I will be waving every time you leave
Oh, I am you
The care, the love, the memories
We are the story of one

Father
I am always close to you
I will be waving every time you leave
Oh, I am you
The care, the love, the memories
You are forever in me

Our Decades in the sun (Nightwish)

Por fim, agradeço a uma pessoa muito, muito especial em minha vida, que me incentiva, me questiona, me ajuda de todas as formas possíveis e impossíveis, entende ou tenta ao máximo entender minhas loucuras, meu grande amigo, meu companheiro de vida, o melhor parceiro de campo que existe, meu amor Carlos Benhur Kasper. Durante esses quase 10 anos de companheirismo☺, cresci e aprendi muito contigo, obrigada por me aceitar e respeitar como sou.

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Sumário

Resumo

Peixes de água doce apresentam muitas vezes uma estrutura filogeográfica marcante, fortemente associada com as mudanças históricas e ecológicas do ambiente aquático. Tem sido sugerido que os padrões atuais de distribuição da fauna de peixes em rios de água doce das drenagens costeiras do Brasil espelhem conexões passadas ou presentes entre estas drenagens. Sendo assim, diferentes condições ecológicas em uma mesma drenagem podem agir como barreiras permeáveis à dispersão e ao fluxo gênico. Estudos anteriores reconheceram dois componentes espaciais discretos para a ictiofauna das bacias costeiras de água doce do sul do Brasil (ecorregião Tramandaí-Mampituba): a ictiofauna das lagoas da planície e a ictiofauna dos rios que drenam pelos vales da encosta da Serra Geral. Duas das três espécies de Stevardiinae endêmicas dessa ecorregião, *Diapoma itaimbe* e *Bryconamericus lethostigmus*, são simpátricas e restritas a ambientes fluviais, sendo ausentes nas lagoas da planície costeira. Assim, essa ecorregião é particularmente interessante para estudar processos filogeográficos e de especiação recentes, sendo ambas as espécies bons modelos de estudo. A fim de testar se as lagoas costeiras podem limitar a dispersão de uma espécie restrita aos rios, descrevemos a história filogeográfica das populações de *D. itaimbe*. Após, abordamos os aspectos filogeográficos de *B. lethostigmus* com uma discussão baseada na comparação entre estas duas espécies simpátricas. Além disso, uma redescrição de *B. lethostigmus* é realizada com adição de dados morfológicos e moleculares. Recentemente ambas as espécies tiveram sua classificação alterada em uma nova proposta de classificação para tribos e gêneros em Stevardiinae baseada em uma filogenia com dados moleculares. Por este motivo, estas espécies são apresentadas com grafias diferentes (*Odontostoechus lethostigmus* e *Cyanocharax itaimbe*) no capítulo 1 (já publicado) em relação aos demais. *Odontostoechus* foi considerado sinônimo junior de *Bryconamericus sensu stricto* e *Cyanocharax,* juntamente com *Hyphessobrycon guarani,* foram atribuídas ao gênero *Diapoma* a fim de definir um gênero monofilético e para ser coerente com a classificação filogenética. Enquanto filogenias moleculares apresentam tanto *Diapoma* como *Cyanocharax* como gêneros parafiléticos mas com as espécies dos dois gêneros formando um único clado, filogenias morfológicas apresentam *Diapoma* e *Cyanocharax* como gêneros distintos em Stevardiinae. Assim, buscou-se esclarecer as relações filogenéticas entre as espécies de *Diapoma* através de análises moleculares e morfológicas comparativas e também da análise simultânea destes dados. Surpreendentemente *Diapoma itaimbe* e *Bryconamericus lethostigmus* apresentaram padrões filogeográficos distintos. O monofiletismo de *Diapoma*

(incluindo *Cyanocharax*) foi recuperado neste estudo em análises separadas, bem como na análise simultânea dos dados moleculares e morfológicos. No entanto, as relações entre as espécies de *Diapoma* foram incongruentes entre as análises morfológicas e moleculares independentes sendo que as filogenias moleculares apresentaram maior suporte para os clados do que as filogenias morfológicas. Já, a análise simultânea mostrou uma resolução para as relações internas de espécies *Diapoma* com suporte superior e com as sinapomorfias morfológicas.

Capítulo Introdutório

INTRODUÇÃO GERAL

Characiformes, Characidae, Stevardiinae

Characiformes é uma das principais ordens de Ostariophysi com 2.492 espécies válidas (Eschmeyer & Fong, 2015) distribuídas na África, América do Sul, América Central e região sul da América do Norte. É um dos grupos mais diversos da ictiofauna neotropical, com mais de 1790 espécies na região (Malabarba & Malabarba, 2014). Dentre os Characiformes, a família mais diversa e com maior complexidade no entendimento das relações de seus componentes é Characidae (Weitzman & Malabarba,1998), com 1.100 espécies válidas (Eschmeyer & Fong, 2015). Além da riqueza de espécies, Characidae apresenta uma morfologia bastante conservada por um longo período de tempo. Por exemplo, Weiss *et al*. (2012) registram fósseis de Characidae com morfologia essencialmente moderna para o Eoceno-Oligoceno. Tal conservação morfológica aparentemente explica o elevado número de homoplasias entre as formas modernas o que dificulta o estabelecimento de relações baseadas somente em filogenias morfológicas (Mirande, 2010; Malabarba *et al*., 2012; Thomaz *et al*.,2015). Devido à falta de hipóteses consistentes acerca das relações entre os diversos grupos de Characidae, Lima e 18 colaboradores passaram a tratar desde 2003 cerca de 2/3 dos membros da família como *incertae sedis* em Characidae (Lima *et al*., 2003).

A família foi redefinida recentemente de modo a incluir somente aqueles representantes de Characidae (*sensu lato*) que não possuem o osso supraorbital (Malabarba & Weitzman, 2003; Mirande, 2010). Estes representantes de Characidae (*sensu stricto*) formam um grupo monofilético suportado por esta sinapomorfia que é igualmente recobrada em filogenias moleculares (Javonillo *et al*., 2010; Oliveira *et al*., 2011).

Filogenias moleculares recentes tem suportado o reconhecimento de três grandes grupos em Characidae *sensu stricto*, denominados informalmente de clados A, B e C (Javonillo *et al*., 2010; Oliveira *et al*., 2011). O primeiro, proposto e nomeado originalmente como Clado A por Malabarba & Weitzman (2003) foi definido pela presença de dois raios não ramificados e oito raios ramificados na nadadeira dorsal e quatro dentes na série interna do premaxilar. Este clado hipotético incluiu em sua definição original, além da subfamília Glandulocaudinae Eigenmann, 1914 *sensu* Weitzman & Menezes (1998) com 19 gêneros, outros 19 gêneros classificados como *incertae sedis* por Lima *et al*. (2003) e que previamente eram classificados em Cheirodontinae e Tetragonopterinae *sensu* Géry (1977) além do gênero *Cyanocharax*, recém descrito no mesmo estudo (Thomaz *et al*., 2015). Posteriormente,

Weitzman *et al*. (2005) restringiram Glandulocaudinae à tribo Glandulocaudini, transferindo as seis tribos restantes de Glandulocaudinae (Weitzman & Menezes, 1998) para a subfamília Stevardiinae.

O monofiletismo do "Clado A", incluindo Glandulocaudinae, Stevardiinae *sensu* Weitzman *et al*. (2005), e os demais gêneros *incertae sedis* (Lima *et al*. 2003) foi corroborado por estudos morfológicos (Mirande, 2010; Baicere-Silva *et al*. 2011; Ferreira *et al*., 2011; Mirande *et al*., 2013) e também moleculares (Calcagnotto *et al*., 2005; Javonillo *et al*., 2010; Oliveira *et al*., 2011; Thomaz *et al*., 2015) desenvolvidos posteriormente.

Após a definição original do Clado A houve a adição de dez gêneros, totalizando 48 gêneros. Mirande (2009), em uma filogenia morfológica, encontrou Stevardiinae *sensu* Weitzman *et al*. (2005) parafilético com Glandulocaudiinae. Desta forma, um conceito mais abrangente de Stevardiinae foi proposto por Mirande (2009) englobando todos os membros do Clado A, sendo Glandulocaudinae reduzido a uma tribo monofilética (Glandulocaudini) dentro de Stevardiinae. Stevardiinae *sensu* Mirande (2009) foi diagnosticado com base em três sinapomorfias: a presença de oito raios ramificados na nadadeira dorsal; a ausência do ramo epiphyseal do canal supraorbital; e a presença de nove pterigióforos na nadadeira dorsal.

A mais recente e abrangente contribuição para o conhecimento da filogenia do grupo foi proposta por Thomaz *et al*. (2015) através da análise de três genes mitocondriais e quatro genes nucleares, redefinindo as relações internas de Stevardiinae e propondo o rearranjo de suas espécies em 46 gêneros e sete tribos, sendo que apenas uma (Glandulocaudini) apresenta composição idêntica àquela proposta por Weitzman *et al*. (2005).

O gênero *Diapoma*

O gênero *Diapoma* Cope, 1894, inicialmente monotípico, foi originalmente diagnosticado pela modificação do aparato opercular observada na espécie tipo, *Diapoma speculiferum* Cope, 1894. Mais tarde, *Glandulocauda terofali* Géry, 1964 foi incluída em *Diapoma*, alterando a diagnose do gênero. Weitzman & Fink (1985) consideraram o compartilhamento de estruturas e disposição das escamas no órgão caudal como diagnósticos do gênero *Diapoma*. Com base na similaridade do órgão caudal entre *Acrobrycon* Eigenmann & Pearson, 1924, *Diapoma* e *Planaltina* Bohlke, 1954, Weitzman *et al*. (1988) incluíram os três gêneros na tribo Diapomini. Finalmente, mais duas espécies foram descritas para *Diapoma* (*D. pyrrhopteryx* Menezes & Weitzman, 2011 e *D. thauma* Menezes & Weitzman, 2011) por Menezes & Weitzman (2011). Esses autores também sugeriram que o gênero se

mantivesse na tribo Diapomini, subfamília Stevardiinae, até que estudos filogenéticos mais completos fossem realizados no grupo.

O gênero *Cyanocharax* Malabarba & Weitzman (2003) foi proposto como pertencente ao grupo monofilético de caracídeos denominado Clado A, embora não houvesse caracteres exclusivos ou sinapomorfias diagnósticas do gênero. Neste mesmo estudo, os autores descreveram seis novas espécies (*Cyanocharax alegretensis* Malabarba & Weitzman 2003, *C. dicropotamicus* Malabarba & Weitzman 2003, *C. itaimbe* Malabarba & Weitzman 2003, *C. lepiclastus* Malabarba, Weitzman & Casciotta, *C. macropinna* Malabarba & Weitzman, 2003, and *C. tipiaia* Malabarba & Weitzman 2003) e apresentam uma nova combinação para *C. alburnus* (Hensel 1870). *Cyanocharax macropinna*, espécie tipo do gênero, foi posteriormente considerada por Malabarba *et al*. (2004) sinônimo júnior de *Hyphessobrycon melanopleurus uruguayensis* Messner, 1962, uma espécie descrita em uma série mimeografada e de circulação restrita (Boletin de la Asociación Latinoamericana de Ictiólogos y Herpetólogos), renomeada em nova combinação como *Cyanocharax uruguayensis*. Mais tarde, Casciotta *et al*. (2012) descreveram mais uma nova espécie, *Cyanocharax obi* Casciotta *et al*., 2012.

A filogenia morfológica de Characidae proposta por Mirande (2010) recobra *Cyanocharax e Diapoma* como gêneros distintos em Stevardiinae (Fig. 1) e não proximamente relacionados. Javonillo *et al*. (2010), em uma análise molecular, encontraram tanto o gênero *Cyanocharax* como *Diapoma* como parafiléticos, porém com as espécies dos dois gêneros formando um clado único (Fig. 2). Posteriormente, Casciotta *et al*. (2012), em uma filogenia com dados moleculares, também agruparam *Cyanocharax* e *Diapoma* em um mesmo clado, tornando *Cyanocharax* parafilético com a inclusão de *Diapoma* (Fig. 3).

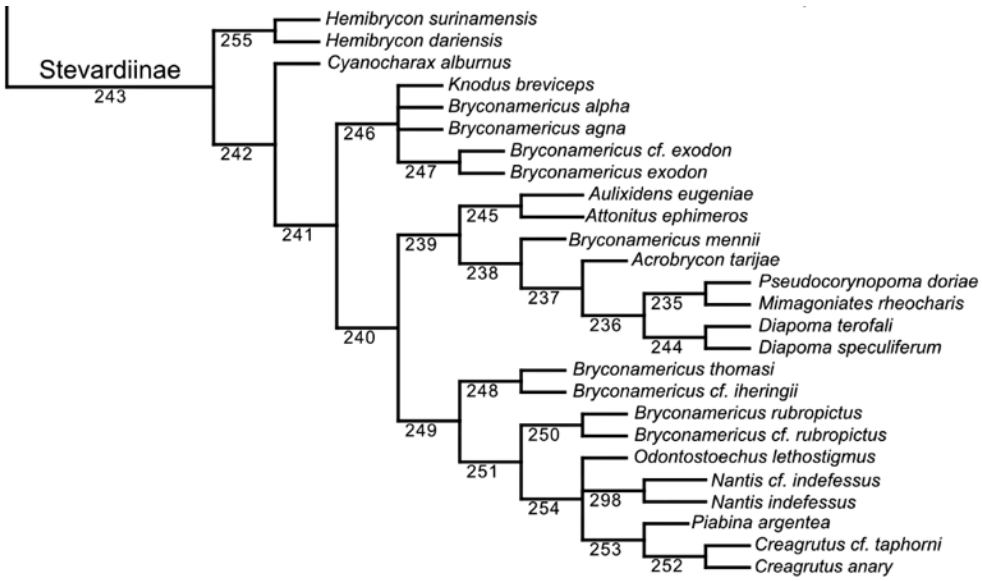

Figura 1. Clado Stevardiinae da filogenia morfológica de Mirande (2010).

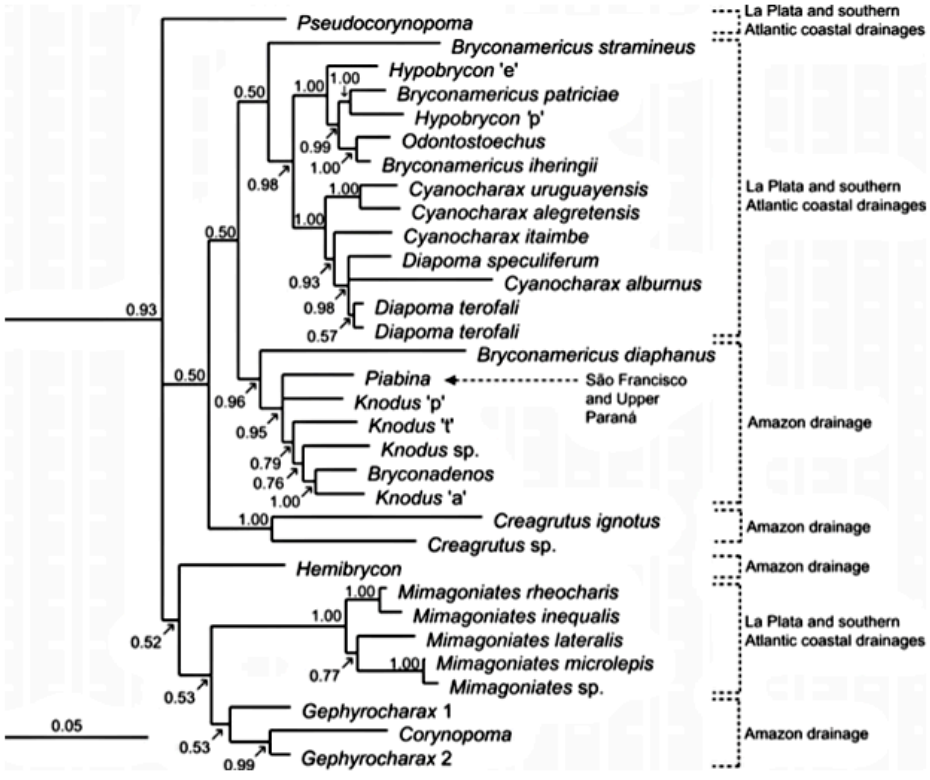

Figura 2. Clado A na filogenia molecular de Javonillo *et al*. (2010).

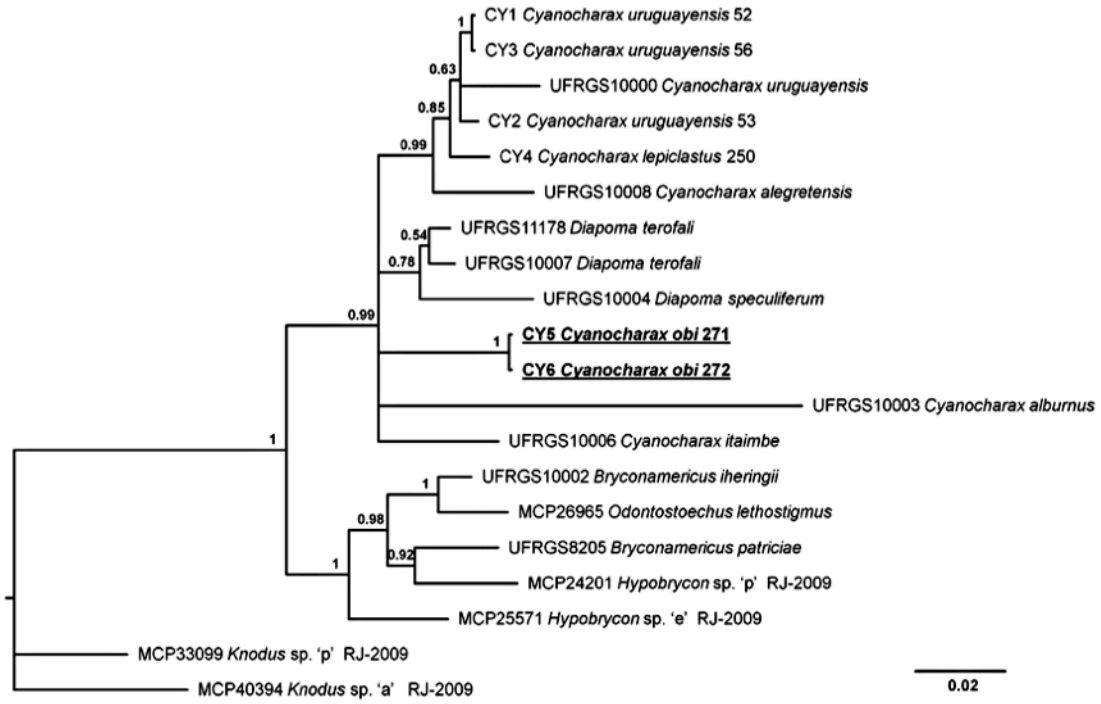

Figura 3. Filogenia molecular do gênero *Cyanocharax* de Casciotta *et al*. (2012).

Recentemente, Thomaz *et al*. (2015) rejeitaram fortemente o monofiletismo de *Cyanocharax* através de testes de topologia com base em dados moleculares. Neste estudo Thomaz *et al*. (2015) propuseram uma nova classificação para tribos e gêneros em Stevardiinae, na qual a tribo Diapomini foi radicalmente reformulada, e todas as espécies de *Cyanocharax*, além de *Hyphessobrycon guarani* Mahnert & Géry (1987), foram atribuídas ao gênero *Diapoma* a fim de definir um gênero monofilético coerente com uma classificação filogenética. Embora não tenha sido feita uma análise conjunta com caracteres morfológicos, os autores propõem como sinapomorfia para este clado o número de raios da nadadeira pélvica (i + 6) diferindo dos demais gêneros em Stevardiinae (i + 7). Esta característica, porém, também é compartilhada com o gênero *Planaltina* o qual não foi analisado por Thomaz *et al*. (2015). Atualmente, com a nova proposta de classificação feita por Thomaz *et al*. (2015), *Diapoma* apresenta treze espécies válidas que estão distribuídas nas bacias hidrográficas do sul da América do Sul, estando presentes na bacia do rio Paraná, Uruguai, Laguna dos Patos e nas drenagens costeiras do Atlântico (Eschmeyer & Fong, 2015).

A região costeira e a ictiofauna

A costa Atlântica do Brasil vem sendo formada por múltiplos processos geológicos desde a ruptura do Gondwana (~ 180 milhões de anos atrás), incluindo elevação de megadomes e eventos de erosão (Ribeiro, 2006). Mais recentemente na escala de tempo, as oscilações climáticas durante o Pleistoceno (~ 2,6 milhões de anos atrás) desempenharam um papel importante afetando esta região, devido aos efeitos das mudanças do nível do mar que impactaram conexões entre rios (Weitzman *et al*., 1988; Lundberg *et al*., 1998) e a mudanças na extensão da cobertura da Mata Atlântica (Carnaval & Moritz, 2008; Carnaval *et al*., 2009).

Nos últimos 500 mil anos, ciclos de mudanças no nível do mar foram importantes para caracterizar a presente fisionomia da planície costeira. O recuo do nível do mar resultou na formação de várias lagoas ao longo da planície que interligaram rios previamente isolados que drenam das montanhas da Serra Geral ao mar (Villwock, 1984; Villwock & Tomazelli, 1995). Esses rios formam uma série de bacias hidrográficas geralmente isoladas, conhecidas como drenagens costeiras do sudeste do Brasil. Tal isolamento histórico contribuiu para a evolução de um número significativo de espécies endêmicas de peixes e outros organismos (plantas, mamíferos, rãs, etc.), fazendo das drenagens costeiras e da Mata Atlântica brasileira, um dos maiores *hotspots* da biodiversidade no mundo (Myers *et al*., 2000). Para peixes de água doce, o endemismo em relação à área é um dos mais altos da região Neotropical (Vari, 1988; Weitzman *et al*., 1988; Bizerril, 1994; Buckup, 2011).

A ecorregião Tramandaí-Mampituba (unidade 335 - Abell *et al*., 2008) é um sistema de três pequenas drenagens isoladas situada na porção sul da região costeira do Brasil. Esta região é identificada como uma área de alto endemismo de espécies de peixes devido aos padrões de distribuição congruentes compartilhados por várias espécies apenas encontradas nestes três drenagens (Malabarba & Isaia, 1992; Reis & Schaefer, 1998). Das nove espécies de peixes exclusivas dessa ecorregião, três são Stevardiinae: *Diapoma itaimbe* (Malabarba & Weitzman, 2003) *Bryconamericus lethostigmus* (Gomes, 1947) e *Mimagoniates rheocharis* Menezes & Weitzman, 1990.

Os peixes de água doce frequentemente exibem estrutura filogeográfica fortemente associada a mudanças históricas e ecológicas no ambiente aquático (Bermingham & Avise, 1986; Bernatchez & Wilson, 1998; Rundle *et al*., 2000; Waters *et al*., 2007). Desta forma, tem sido sugerido que os padrões atuais de distribuição da fauna de peixes em rios de água doce das drenagens costeiras do sudeste do Brasil espelhem conexões passadas ou presentes entre

estas drenagens (Weitzman *et al*., 1988;. Lundberg *et al*., 1998). Assim, essa ecorregião é particularmente interessante para estudar processos filogeográficos e de especiação recentes.

Para testar a hipótese de que espécies de distribuição simpátrica teriam padrões filogeográficos semelhantes, estudamos duas das três espécies de Stevardiinae que são endêmicas da ecorregião Tramandaí-Mampituba, *Diapoma itaimbe* e *Bryconamericus lethostigmus*. Estas duas espécies simpátricas são estritamente restritas a ambientes fluviais, sendo ausentes nas lagoas da planície costeira.

Assim, no primeiro capítulo abordamos os aspectos filogeográficos de *D. itaimbe* discutindo sobre a hipótese de que as lagoas costeiras ou possíveis conexões entre as drenagens na planície costeira possam agir como barreira para a dispersão entre populações de peixes estritamente adaptadas a ambientes fluviais.

No segundo capítulo abordamos os aspectos filogeográficos de *B. lethostigmus* com uma discussão baseada na comparação entre estas duas espécies simpátricas. Além disso, uma redescrição de *B. lethostigmus* foi realizada com adição de dados morfológicos e moleculares. A caracterização morfológica da espécie permitiu uma discussão das implicações filogenéticas e possíveis homologias com outros Stevardiinae.

Finalmente, no terceiro capítulo realizamos uma filogenia gerada com dados morfológicos e moleculares a fim de testar o monofiletismo do gênero *Diapoma*, elucidar as relações entre as espécies e verificar possíveis sinapomorfias para o grupo.

OBJETIVOS

O presente trabalho teve como objetivo geral formular hipóteses da história evolutiva e relações entre as populações de duas espécies simpátricas, *Diapoma itaimbe* e *Bryconamericus lethostigmus*, e verificar o monofiletismo e as relações filogenéticas das espécies de *Diapoma*.

Os objetivos específicos foram:

a. Compreender a história evolutiva das duas espécies simpátricas *D. itaimbe* e *B. lethostigmus*, através de abordagem filogeográfica e filogenética;
b. Fazer a redescricão da espécie *B. lethostigmus* com o acréscimo de dados morfológicos e moleculares;

c. Propor uma hipótese de relações filogenéticas entre as espécies de *Diapoma* a partir de caracteres morfológicos e moleculares.

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Capítulo I

Aviso

O Capítulo I já está publicado no periódico Zoologica Scripta.
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Hirschmann, A. Thomaz, A. T., Malabarba, L. R., Fagundes, N.J.R. (2015). Riverine habitat specificity constrains dispersion in a Neotropical fish (Characidae) along Southern Brazilian drainages. *Zoologica Scripta*, 44, 374-382.

Neste capítulo onde está *Cyanocharax* lê-se *Diapoma*. A nomenclatura não foi alterada na tese devido ao artigo já estar publicado.

Zoologica Scripta

KUNGL. VETENSKAPS-AKADEMIEN
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Riverine habitat specificity constrains dispersion in a Neotropical fish (Characidae) along Southern Brazilian drainages

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Freshwater fishes often display a marked phylogeographic structure strongly associated with historical and ecological changes in the aquatic environment. Different ecological conditions in the same river drainage may act as permeable barriers to dispersion and gene flow. Previous studies recognized two discrete spatial components for the ichthyofauna in the freshwater coastal drainages of southern Brazil: the lowland fish fauna in the lagoons and the fish fauna of the rivers flowing in the valleys. In order to test if the coastal lagoons may limit the dispersion of a riverine species, we describe the phylogeographic structure among populations of *Cyanocharax itaimbe*, a species endemic to this region. We analysed 55 specimens characterized for two mitochondrial and one nuclear genes. Sequences were analysed using gene trees and species tree approaches, together with standard population genetics methods. Molecular analyses indicated three evolutionary groups which diverged from each other between an estimated 1,600,000 and 450,000 years before the present. However, two currently isolated river systems share the same evolutionary clade, whereas a single drainage contains two different lineages. Our results indicate strong genetic structure among populations along with generally conserved morphology. The strong genetic structure among populations living in the same drainage system may be explained by ecological differences between lagoons and rivers (or palaeochannels) that act as barriers to dispersion.

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Introduction

Freshwater fishes often display marked phylogeographic structure strongly connected to historical and ecological changes in the aquatic environment and landscapes (Bermingham & Avise 1986; Bernatchez & Wilson 1998; Rundle *et al.* 2000; Waters *et al.* 2007). In general, these organisms depend directly on connections between river basins for dispersion. Thus, a strong relationship is expected between the history of the basins and their associated ichthyofauna. In other words, for freshwater fishes, phylogeography is integrally linked to the landscape and to the landscape history (Avise 2000, 2009).

The landscape of the Brazilian Atlantic coastal region was formed by multiple geological processes since the breakup of the Gondwana (~180 million years ago - Ma), including megadome uplift and erosion events (Ribeiro 2006). On a more recent timescale, climatic oscillations during the Pleistocene (~2.6 Ma) played a major role affecting this region due to the effects of sea level changes which impacted connections between rivers (Weitzman

et al. 1988; Lundberg *et al.* 1998) and shifts in the extent of the Atlantic Rainforest cover (Carnaval & Moritz 2008; Carnaval *et al.* 2009). In the latest 0.5 Myr, cycles of sea level changes were important for the present physiognomy of the Southern Coastal Plain. Sea level retreat resulted in the formation of several lagoons along the long coastal plain and interconnected previously isolated rivers draining from the Serra Geral highlands to the sea (Villwock 1984; Villwock & Tomazelli 1995; Fig. 1). These rivers form a series of isolated hydrographic basins, collectively known as Coastal Drainages of Southeastern Brazil (CDSEB). Smaller isolated basins are separated from the large inland drainages by the scarped, mountainous landscapes of the eastern margin of the Brazilian mountain ranges that extend from southern to southeastern Brazil (Ribeiro 2006). Such historical isolation contributed to the evolution of a significant number of endemic species of fishes and other organisms (plants, mammals, frogs, etc.). This factor makes the CDSEB, and the presence of the Brazilian Atlantic Rainforest, one of the greatest hotspots of biodiversity in the world (Myers *et al.* 2000). For freshwater fishes, the endemism in relation to area is one of the highest in the Neotropical region (Vari 1988; Weitzman *et al.* 1988; Bizerril 1994; Buckup 2011).

A system of three small isolated drainages characterizes the southern portion of the CDSEB: the Rio Tramandaí, Rio Mampituba and Rio Araranguá, which comprise the Tramandaí-Mampituba Freshwater Ecoregion (unit 335 - Abell *et al.* 2008; Fig. 1). This region is identified as an area of high endemism for species of fishes because of the congruent distributional patterns shared by several species solely found in these three drainages (Malabarba & Isaia 1992; Reis & Schaefer 1998). One of these river systems, the Rio Tramandaí, exhibits a distinctive feature: two rivers in this basin, the Rio Maquiné and Rio Três Forquilhas (Fig. 1) flow in isolation in different valleys of the Serra Geral, but are connected near their mouths by freshwater lagoons (Lagoa dos Quadros and Lagoa Itapeva) and small coastal plain channels. The other two rivers (Rio Mampituba and Rio Araranguá) run in complete isolation and then empty into the sea. These rivers are small and shallow with clear, cold waters, a rapid flow and rocky bottoms.

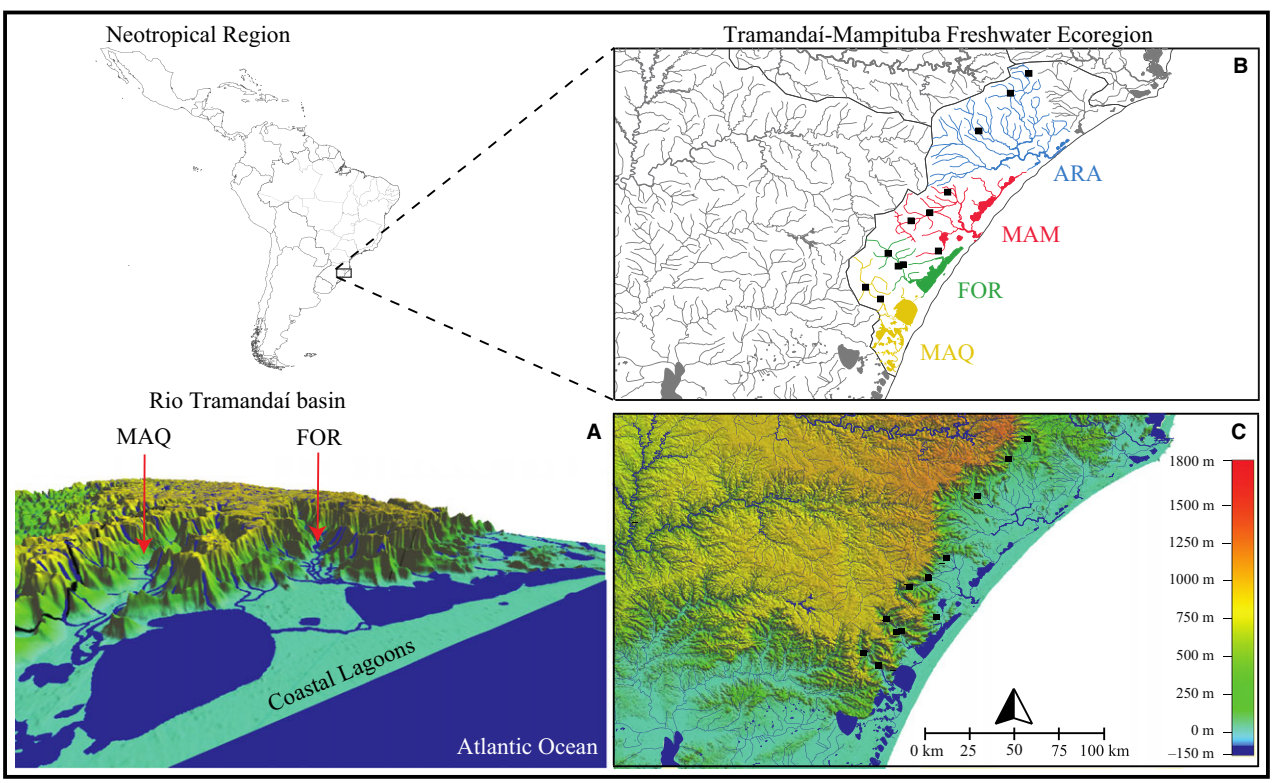

Fig. 1 Geographical distribution of *Cyanocharax itaimbe*. —A. 3D map highlighting of the Rio Tramandaí drainage demonstrating that the Rio Maquiné and Três Forquilhas are isolated upstream but connected downstream through freshwater lagoons and small channels in the coastal plain. The slope has been exaggerated for better visualization. —B. Tramandaí-Mampituba Freshwater Ecoregion, the region of focus in this study, is shown with different colours for each river basin/subbasin in the ecoregion; squares represent sampled localities of *C. itaimbe*. —C. Elevation map with sampling sites.

Conversely, the freshwater lagoons connecting some of these rivers are large, circular/elliptical, shallow and sandy bottomed, with slow water flow. For example, both lagoons (Lagoa dos Quadros and Lagoa Itapeva) have a maximum depth of about 3.5 m, whereas their surface areas are 119 km^2 and 95.16 km^2, respectively (Schwarzbold & Schäfer, 1984). These characteristics result in higher turbidity and temperature in the lagoon than in the rivers.

The present configuration of the coastal plain and the formation of these freshwater lagoons are relatively young. According to Schwarzbold & Schäfer (1984) the formation of Lagoa dos Quadros and Lagoa Itapeva dates to about 5 thousand years ago (ka). The Holocene transgression that covered the coastal plain with sandy barriers is no more than 5 ka in age. The formation of these Holocene sandy barriers was the final episode of sequential barrier formation results from sea level variations during the Quaternary. These variations culminated in the generation of the current landscape of coastal lagoons that characterizes the study area (Villwock & Tomazelli 1995; Tomazelli *et al.* 2000).

Based on the differences of the ichthyofauna between rivers and lagoons of this region Malabarba & Isaia (1992) recognized two discrete components in fish fauna of in the Rio Tramandaí drainage: the lowland fish fauna present in the lagoons and the riverine fish fauna in the valleys. For instance, 16 species found in the rivers have never been captured in the lagoons (Malabarba *et al.* 2013). Thus, these species are isolated within each river basin despite being part of a common drainage.

It has been suggested that the current patterns of distribution of this fish fauna in CDSEB rivers reflect past or present freshwater connections among these drainages (Weitzman *et al.* 1988; Lundberg *et al.* 1998). To test this hypothesis, we studied *Cyanocharax itaimbe* Malabarba & Weitzman 2003 (Fig. 2), which is endemic to the Rio Tramandaí, Rio Mampituba and Rio Araranguá drainages. *Cyanocharax itaimbe* lives in strict riverine environments with clear, cold waters over rocky substrates, and is absent from coastal lagoons (Malabarba & Weitzman 2003). If recent lowland freshwater connections were used by this species, the phylogeographic structure among drainages should be low. On the other hand, if *C. itaimbe* was always limited to riverine environments due to its ecological specialization, a strong phylogeographic structure is expected.

Fig. 2 *Cyanocharax itaimbe*, male, 52.1 mm SL (UFRGS 16499) collected in a tributary of the rio Três Forquilhas (FOR), Itati, Rio Grande do Sul state, Brazil.

Although the isolated populations of *C. itaimbe* are morphologically similar, Malabarba & Weitzman (2003) described significant differences in mean and median values of branched anal-fin ray counts among them, suggesting some population structure among drainages. These authors recognized two or three groups based on the mean and median of branched anal-fin rays. In the two-group classification, the population from Rio Araranguá, with a low number of branched anal-fin rays (median = 23, mean = 22.9), is distinguished from the other populations (Rio Mampituba; Rio Três Forquilhas; Rio Maquiné; median = 24; 25; 25 and mean = 23.9; 24.6; 24.7 respectively). In the three-group classification, the population from Rio Mampituba is further distinguished from the others (Rio Três Forquilhas and Rio Maquiné) also by different count in branched anal-fin rays.

Based on the above cited historical landscape features and the morphological evidence, we hypothesize that, if the lagoons are acting as barriers to the recent dispersion between populations of this specialized riverine fish, we predict that this species will show significant genetic structure not only among isolated drainages (Rio Tramandaí, Rio Araranguá and Rio Mampituba), but also between the rivers within the Rio Tramandaí basin (Rio Maquiné and Rio Três Forquilhas). Otherwise, if this characid is able to use the lagoons for dispersion between the populations in these two rivers in the Tramandaí drainage, a lack of structure between these two populations would be predicted. Given that the overall biogeographic pattern exhibited by *C. itaimbe* is shared with several species, the results provide initial alternative evidence for the effect of freshwater lagoons on riverine dispersion along the southern Atlantic coast.

Materials and methods

Tissue samples from 55 specimens of *Cyanocharax itaimbe* were collected throughout its distribution and maintained in 96% ethanol in the fish collection at the Department of Zoology, Universidade Federal of Rio Grande do Sul (UFRGS). Samples include individuals from all four rivers systems in the range of this species (Rio Araranguá – ARA; Rio Mampituba – MAM; Rio Três Forquilhas – FOR; and Rio Maquiné – MAQ). Tissue samples from closely related species from the UFRGS fish collection were used as outgroups: one individual each of *Cyanocharax alburnus* (Hensel, 1870), *C. alegretensis* Malabarba & Weitzman, 2003; *C. dicropotamicus* Malabarba & Weitzman 2003;

C. uruguayensis (Messner, 1962), *Diapoma speculiferum* Cope, 1894 and *D. terofali* (Géry, 1964) (Fig. 1, Table 1). *COI* sequences for *C. alegretensis*, *C. uruguayensis* and *D. terofali* were obtained from GenBank (FJ749047.1, FJ749049.1 and FJ749052.1; Javonillo *et al.* 2010).

DNA extraction from tissues followed a modified salt precipitation protocol (Medrano *et al.* 1990). For each sample we used PCR to amplify two mitochondrial genes: cytochrome oxidase subunit I (*COI*) and NADH dehydrogenase 2 (*ND2*); and three nuclear genes: SH3 and PX domain-containing 3-like protein (*SH3PX3*), S7 ribosomal protein intron 2 (*S7*2) (Cooke & Beheregaray 2007) and myosin heavy chain 6 (*Myh6*) (Li *et al.* 2007). PCRs were carried out in 20 μL reactions containing 10-50 ng DNA, 0.2 μM of each primer, 0.2 mM of each dNTP, 1× Buffer, 1.5 μM $MgCl_2$ and 1U Platinum Taq DNA polymerase (Invitrogen, São Paulo, SP, Brazil). PCR conditions and primers are presented in Appendix S1 Table S1. 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.

The mitochondrial coding genes *COI* and *ND2* were concatenated for both phylogeographic and phylogenetic analyses whereas the nuclear genes were analysed separately or by using species-tree methods without concatenation (see below). Forward and reverse chromatogram reads were assembled and visualized using Geneious 5.6.7 – trial version (Drummond *et al.* 2012). The consensus sequences were automatically aligned using the software CLUSTALW (Thompson *et al.* 1994) with default parameters and implemented in BIOEDIT 7.1.3.0 (Hall 1999). We used the software package Phase version 2.1 (Stephens *et al.* 2001; Stephens & Scheet 2005) to estimate haplotypes for the nuclear gene *SH3PX*3. Basic statistics, such as nucleotide (π) and haplotype diversity (hd) as well as neutrality tests were calculated in the software DNASP v. 5 (Librado & Rozas 2009).

We also constructed haplotype networks with the median-joining method (MJN) (Bandelt *et al.* 1999) using the program network 4.1.0.8 (www.fluxus-engineering.com). Calculation of F-statistics (Φ_{ST}) and Analysis of Molecular Variance (AMOVA) were carried out in the program ARLEQUIN 3.5 (Excoffier *et al.* 2005). For these analyses, individuals sampled in the same river drainage were merged into a single population to quantify the amount of genetic structure amongst them. To test if MAQ and FOR may represent isolated populations, even though the coastal lagoons connect them, we kept them as two distinct populations. Thus, the analysis considered one group with four populations.

Phylogenetic relationships among populations of *C. itaimbe* and between *C. itaimbe* and outgroups were inferred by Bayesian inference (BI) and Maximum Parsimony (MP). Separate MP analyses for each partition (mitochondrial and nuclear) were conducted with TNT (Goloboff *et al.* 2008) using a traditional search with 200 replicates and tree bisection reconnection (TBR) algorithm branch swapping and 20 random taxon addition replicates. All characters were unordered with character transformations equally weighted. Clade robustness was assessed using 1000 bootstrap pseudoreplicates with the same search as above. BI

Table 1 Species, populations, voucher specimens and geographical coordinates for the tissues samples used in this study. All samples belong to UFRGS fish collection (Universidade Federal do Rio Grande do Sul)

Species	Population (Drainage)	Voucher	Lat. (S)	Long. (W)
Cyanocharax itaimbe	Maquiné/Tramandaí	UFRGS 12084/TEC1231A, C, E, F, H	29°39′07″	50°12′34″
	Maquiné/Tramandaí	UFRGS 12634/TEC1369A, B, E, F, G, H	29°35′02″	50°16′51″
	Três Forquilhas/Tramandaí	UFRGS 12585/TEC1261A, B, E, F, H	29°29′09″	50°07′15″
	Três Forquilhas/Tramandaí	UFRGS 12587/TEC1263A, C, E, F, G, H	29°29′01″	50°05′59″
	Três Forquilhas/Tramandaí	UFRGS 16517/TEC2845A, B, C	29°25′36′′	50°10′44′′
	Mampituba	UFRGS 12534/TEC1236E	29°14′49″	50°4′12″
	Mampituba	UFRGS 12600/TEC1300A, B, C, D, E	29°23′53″	49°55′04″
	Mampituba	UFRGS 12620/TEC1355A, C, D, E, F, G	29°11′41″	49°57′55″
	Mampituba	UFRGS 12635/TEC1370B, D, E, F, G	29°08′29″	49°53′35″
	Araranguá	UFRGS 12625/TEC1360A, E, H	28°47′53″	49°42′57″
	Araranguá	UFRGS 12627/TEC1362A, B, C, D, E, F, H	28°30′39″	49°28′44″
	Araranguá	UFRGS 16567/TEC2899A, B, C	28°36′35′′	49°33′16.2′′
Cyanocharax dicropotamicus	Laguna dos Patos	UFRGS 12727/TEC1465A	28°56′07″	51°26′35″
Cyanocharax alburnus	Lagoa dos Quadros/Tramandaí	UFRGS 12087/TEC1234A	29°40′35″	50°08′29″
Cyanocharax alegretensis	Uruguay	UFRGS 10008/TEC714	28°17′/33°24′	53°39′/57°36′
Cyanocharax uruguayensis	Uruguay	UFRGS 10000/TEC46	31°6′58″	55°24′56″
Diapoma speculiferum	Laguna dos Patos	UFRGS 12388/TEC692	30°8′53.35″	52°2′48.58″
Diapoma terofali	Uruguay	UFRGS 10007/TEC15	30°12′42.80″	55°3′17.50″

using two gene trees (mt- and nDNA) and species tree analysis were implemented in BEAST 1.7.5 (Drummond & Rambaut 2007). This program was also used to estimate dynamics of population size fluctuation over time for *C. itaimbe*, based on the mtDNA dataset under the Extended Bayesian Skyline Plot (EBSP) method (Heled & Drummond 2008). The mtDNA data set was analysed assuming an evolutionary rate of 0.01/site/Myr (Bermingham *et al.* 1997; Reeves & Bermingham 2006; Ornelas-García *et al.* 2008) and a strict clock model, which is a generally well-justified analysis within a species or among a few closely related species (Li & Drummond 2012). The evolutionary rate for *SH3PX3* was calibrated based on the mtDNA rate. We used the Bayesian Information Criterion in PartitionFinder (Lanfear *et al.* 2012) to assume the HKY+I substitution model for *COI*, TN93+I for *ND2* and HKY for *SH3PX3*. For the gene tree estimation, we used 10 million MCMC steps for both mtDNA and *SH3PX3*, while 100 million MCMC steps were performed for species tree estimation. For the EBSP method, the search lasted 50 million MCMC steps. In all cases, samples were collected every 1000 steps and the efficiency of the chain was assessed in Tracer 1.5 with 10% burn-in. We used Bayes Factors (Kass & Raftery 1995) for the species tree analyses to evaluate the model evidence for six different schemes for defining terminals: (i) considering collection sites as terminals (12 terminals); (ii) considering river systems as terminals (four terminals); (iii) considering MAQ+FOR+MAM as one terminal and ARA as a different terminal (two terminals); (iv) considering FOR+MAM as one terminal, MAQ as another terminal and ARA as a third terminal (based in three mtDNA clades (3 terminals); (v) considering the system of three drainages as terminals (three terminals, MAQ+FOR, MAM, ARA); and (vi) considering all *C. itaimbe* populations as a single terminal (one terminal).

Results

A total alignment of 1490 base pairs (bp) was obtained for the mitochondrial genes *COI* (563 bp) and *ND2* (927 bp). The nucleotide and haplotype diversity were 0.014 and 0.97, respectively. A total of 86 polymorphic sites defining 33 different haplotypes for *C. itaimbe* were found. Neutrality tests failed to reject the null hypothesis of no evidence of natural selection and/or constant population size (Tajima's D −0.045, $P > 0.10$; Fu's FS −0.30, $P = 0.23$). In agreement with the results from the neutrality tests, the EBSP approach for mtDNA suggests that *C. itaimbe* maintained a highly effective population size (Ne) over time, suggesting absence of population expansion (Fig. S1 in Appendix S2).

We obtained an alignment of 371 bp and 779 bp for the *S72* gene and *Myh6* gene, respectively, but both of them were not variable in the studied species and, therefore, not used in the analyses. For the *SH3PX3* gene, we obtained an alignment of 700 bp containing seven variable sites which defined nine different haplotypes in *C. itaimbe*. Nucleotide and haplotype diversity were 0.002 and 0.68, respectively, and as with mtDNA the neutrality tests did not indicate violations of the null model (Tajima's D −0.097; $P > 0.10$; Fu's FS −0.29; $P = 0.30$). New sequences generated in this study were submitted to GenBank (KP399679 to KP399736; KP406648 to KP406708; KP636960 to KP637020).

Mitochondrial data showed a strong genetic structure (Fig. 3a), with three well defined groups. The first group consists of haplotypes sampled in populations collected in MAM and FOR. The second group is composed exclusively of haplotypes sampled in MAQ. Finally, the last group consists of haplotypes only observed in ARA. On the other hand, the haplotype network for gene *SH3PX3* (Fig. 3b) showed a central haplotype shared by all populations. Among haplotypes with more restricted geographic distributions, there are interestingly some haplotypes exclusive in ARA, whereas others are shared only between MAM and FOR. MAQ population had only the central haplotype and showed a remarkably low diversity. Overall, both networks appear to be congruent with respect to each other. In agreement with the haplotype networks, the AMOVA (Table 2) shows high isolation among river systems for the mtDNA data ($\Phi_{ST} = 0.85$), while the genetic structure for nDNA was much more modest ($\Phi_{ST} = 0.32$).

Both phylogenetic methods (BI and MP) produced gene trees with similar topologies, and, therefore, we only present results for BI. The Mitochondrial gene tree was congruent with haplotype network and clearly distinguishes three clades within *C. itaimbe* with high posterior probability (PP) values (Fig. S2 in Appendix S2). The first clade is represented by ARA, which is sister to a clade formed by the two other clades, with separate haplotypes from MAQ and MAM+FOR. As expected due to low sequence divergence, the gene tree for *SH3PX3* did not show strong support for any specific clade within *C. itaimbe* and the species was not recovered as a monophyletic group (Fig. S3 in Appendix S2).

The species tree was the one with strongest support (log10 Bayes Factors >1 against one tree and >4 against others) based on the three mtDNA clades (Fig. 4, Table S2 in Appendix S1). This tree shows that MAQ and FOR+MAM are sister clades (PP = 0.99), and that ARA is sister to this clade (PP=0.99). According to the best supported species tree, ARA diverged from the remaining populations in the Pleistocene, around 1.6 Ma (95% Highest Posterior Density (HPD) between 0.7–2.9 Ma). This is very similar to the estimated divergence between two other species, *C. alburnus* and *C. dicropotamicus*, which comprise the sister clade of *C. itaimbe* and whose divergence is esti-

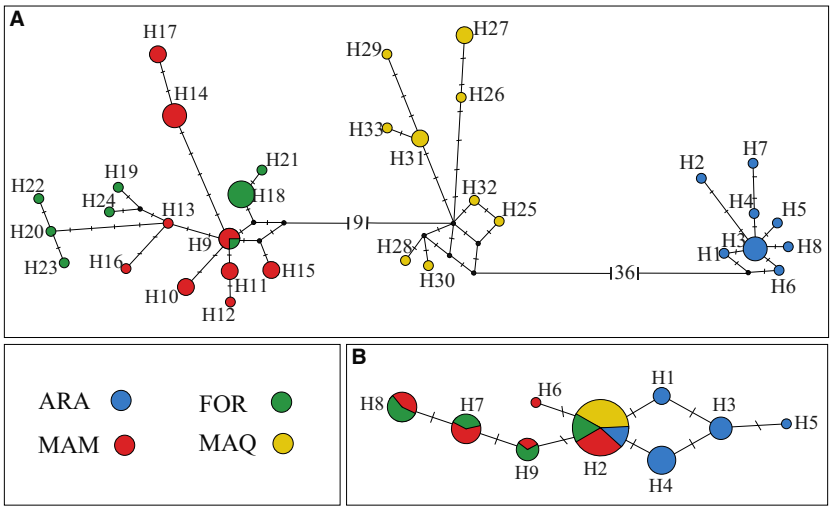

Fig. 3 Median-joining networks among haplotypes of *Cyanocharax itaimbe* samples: (A) inferred by the concatenated mtDNA dataset (*COI+ND2*) and (B) inferred by nDNA (*SH3PX3*). Each circle represents a unique haplotype with circle sizes being proportional to their frequencies. Each colour represents a population as in Fig. 1. Crossed markers indicate the number of mutations between haplotypes.

Table 2 Results from the analysis of molecular variance (AMOVA) among and within *Cyanocharax itaimbe* populations for the mtDNA and nDNA data

	mtDNA			nDNA		
Source of variation	d.f.	% variation	*P*-value	d.f.	% variation	*P*-value
Among populations	3	85.02	<0.001	3	31.58	<0.001
Within populations	51	14.98	<0.001	106	68.42	<0.001
Fixation Index Φ	0.85017		<0.001	0.31581		<0.001

mated to be around 1.8 Ma (95% HPD 0.75–3.00 Ma). Divergence between the remaining clades within *C. itaimbe* is estimated roughly 0.45 Ma (95% HPD 0.20–0.90 Ma). The estimated evolutionary rate for the *SH3PX3* gene relative to the mtDNA rate is as 0.000545/site/Myr (95% HPD 0.000183–0.000936/site/Myr).

Discussion

Our results estimate that *C. itaimbe* from MAQ diverged from the FOR+MAM clade about 450 ka in the Pleistocene, an event synchronous with the initial establishment of the modern coastal plain in southern Brazil. This result does not corroborate the three-group hypothesis of Malabarba & Weitzman (2003) based on morphology. The geomorphological history of this area is well-known and the age of the depositional environments indicates that it has evolved from sediment deposition through a minimum of four successive trans-regressive cycles that occurred during the last 500 ka (Villwock 1984; Villwock & Tomazelli 1995; Buchmann *et al.* 1998). This demonstrates that, besides the potential connections between MAQ and FOR drainages during several marine regressions and the current connection by the freshwater lagoons, the populations of these two rivers remained isolated long-term.

According to Albert & Reis (2011), Neotropical rivers and floodplains often represent dispersal corridors for freshwater fishes. In the case of basins along the coast of Brazil, sea level retreats during glaciation periods would have created temporary connections among presently isolated basins, allowing fishes to disperse between them (Weitzman *et al.* 1988; Lundberg *et al.* 1998). However, such connections may not represent dispersal corridors for ichthyofauna as a whole (Albert & Reis 2011). In the case of species adapted to upstream environments the lowland environments may represent a barrier to gene flow among adjacent drainages. This seems to be the case for *C. itaimbe*. The absence of shared haplotypes between FOR and MAQ, with FOR being the sister clade of MAM, instead of MAQ, indicate that, although FOR and MAQ are currently connected through freshwater lagoons, the latter water bodies act as filters to the dispersion of this species. Past or present lowland river connections cannot be considered the unique feature determining current distribution of freshwater fish lineages along the CDSEB. Instead, the results obtained from *C. itaimbe* confirm that lagoons or possible lowland connections may act as barriers to dispersion between populations of specialized riverine fish. In a similar way, large rivers can act as geographic barriers or natural

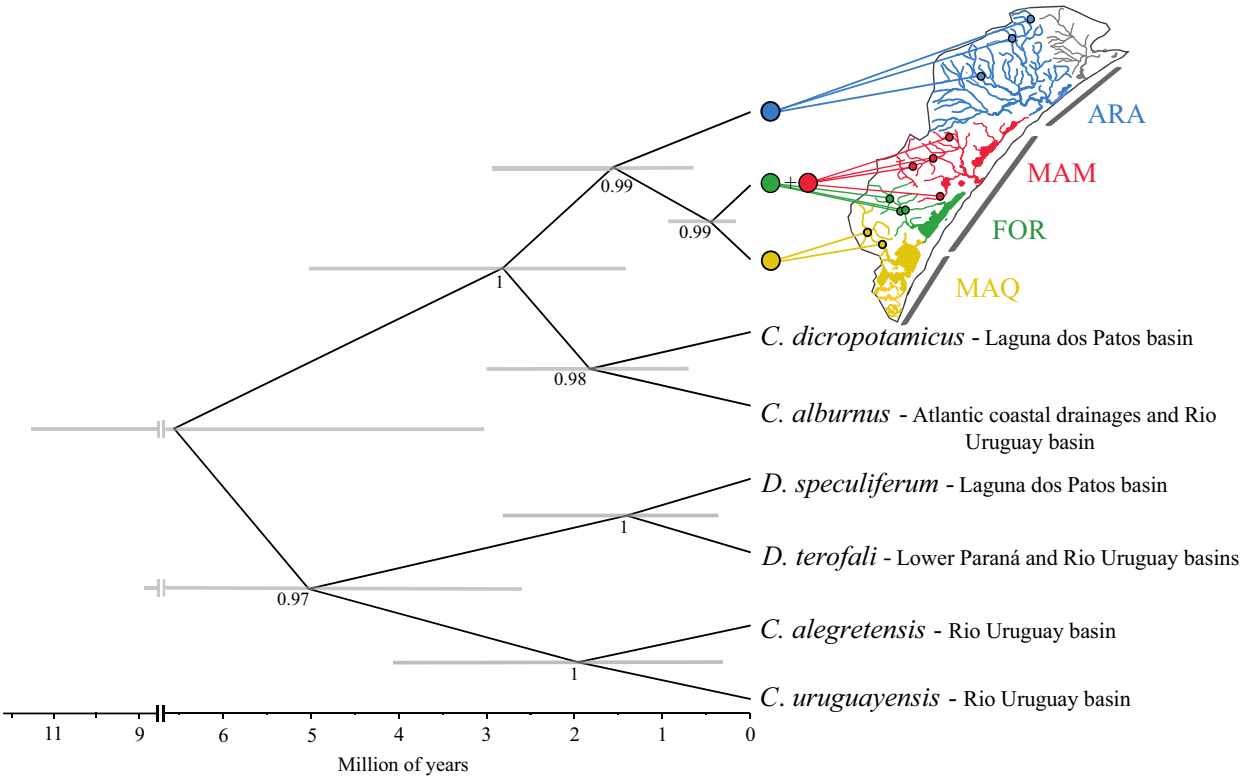

Fig. 4 Species tree with strongest support according to Bayes Factor value. Black numbers on the nodes represent posterior probabilities higher than 0.5 and the grey represents error bars obtained from molecular clock estimate. The colours indicate the sampled population as in Fig. 1.

ecological barriers for fish dispersal processes due to differences in water chemistry or obstacles like waterfalls and rapids (Junk & Soares 2001; Ingenito & Buckup 2007; Torrente-Vilara *et al.* 2011). For example, in the Amazonian region a southern-northern shift in the distribution of fish species and/or genetic diversity can be attributed to the main channel of the Amazon River acting as a physical barrier to dispersal (Hubert & Renno 2006).

Interestingly, in the Rio Tramandaí drainage there is a notable division in the composition of the freshwater fish fauna, with different species found in the major rivers within the Serra Geral Formation and in the lagoons of the coastal plain (Malabarba & Isaia 1992; Malabarba *et al.* 2013). Several species (e.g. *C. itaimbe*, *Deuterodon stigmaturus* Gomes (1947), *Epactionotus bilineatus* Reis & Schaefer 1998; *Hollandichthys taramandahy* Bertaco & Malabarba 2013, *Jenynsia unitaenia* Ghedotti & Weitzman 1995, *Mimagoniates rheocharis* Menezes & Weitzman 1990, *Odontostoechus lethostigmus* Gomes 1947, *Pareiorhaphis hypselurus* (Pereira & Reis 2002), *P. nudulus* (Reis & Pereira 1999), *Rineloricaria aequalicuspis* Reis & Cardoso 2001, and *R. maquinensis* Reis & Cardoso 2001) are restricted to the upland portion of these drainages. These species have smaller ranges than do species occurring in the lowland coastal plain portions of these rivers (e.g. *C. alburnus* (Hensel, 1870), *Jenynsia multidentata* (Jenyns, 1842), *Loricariichthys anus* (Valenciennes, 1836) and *Rineloricaria quadrensis* Reis, 1983). Selective dispersion through coastal rivers or palaeochannels connected with such water bodies along the extended coastal plain during glacial periods acts as dispersal corridors for some species and also as effective freshwater barriers for others. Additional phylogeographic studies comparing patterns found in species from the coastal lagoons and rivers in the Serra Geral will be useful to test this hypothesis. The shared haplotypes between FOR and MAM, despite the present occurrence of these populations in different drainages, may indicate an ancestral relationship between these populations or recent gene flow due to headwater capture, a process still active in the rivers of the coastal region (Ribeiro 2006).

ARA diverged from the remaining *C. itaimbe* populations in the Pleistocene, about 1.6 Ma. Thus, our results corroborate the two-group hypothesis of Malabarba & Weitzman (2003) based on morphology. During the Pleistocene, the Rio Araranguá basin was probably the first of these basins to lose its connection with the neighbouring drainages, with consequent isolation of this population. The low

evolutionary rate of the *SH3PX3* gene, its larger population size compared to mtDNA genes, and the relatively recent divergence time estimated between these populations make the lack of reciprocal monophyly between ARA and the remaining *C. itaimbe* populations unsurprising.

The only morphological difference found between the populations was the anal-fin rays count. The population of ARA significantly differ from the other three populations (MAM, FOR, MAQ), while MAM show a smaller deviation (but still significant) from FOR and MAQ. This morphological character agrees with our molecular data in relation to ARA differing from the other populations. However, the difference between MAM from FOR and MAQ was less significant and does not reflect diverging clades in our mtDNA analysis.

In conclusion, our data support the interpretation that genetic structure found in *C. itaimbe* is explained by ecological factors that impede gene flow between river systems, notwithstanding their current connection by freshwater coastal lagoons, as is the case of MAQ and FOR populations. This also indicates that past lowland river connections between CDSEB due to sea level retreats during glaciation periods may have been selective and may not have represented dispersal corridors for the whole ichthyofauna. Other factors that could have facilitated headwater capture in the upper portion of these rivers may have also played a role, as seen in the case of extensive haplotype sharing between the currently isolated FOR and MAM populations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Additional tables (S1 and S2).

Appendix S2. Additional figures (S1–S3).

Supporting Information

Appendix S1 Additional tables (S1 and S2)

Table S1 Primers used in this study, with their sequence, if 1st or 2nd PCR, references and PCR conditions.

Gene	Primer sequence (liste from 5' to 3')	PCR	Reference	Desnaturation	Cycles	Extension
COI-H2198	TAA AcT TcA ggg TgA ccA AAA AAT cA	1st	Herbert *et al*. (2003)	96°C/1'	40x 94°C/30", 50°C/20", 48°C/5", 46°C/5", 44°C/5", 42°C/5", 40°C/20", 72°C/1'	72°C/3'
COI-L1490	ggT cAA cAA ATc ATA AAg ATA TTg g	1st	Herbert *et al*. (2003)			
ND2-L5216	GGC CCA TAC CCC GRA AAT G	1st	Sorenson *et al*. (1999)	94°C/4'	9x 94°C/30", 57°C-1°C/cycle/1', 72°C/1'30", 40x 94°C/30", 47°C/1', 72°C/1'30"	72°C/5'
ND2-H6313	ACT CTT RTT TAA GGC TTT GAA GGC	1st	Sorenson *et al*. (1999)			
SH3PX3-F461	GTATGGTSGGCAGGAACYTGAA	1st	Li *et al*. 2007	94°C/3'	30x 94°C/30", 55°C/45", 72°C/1'30"	72°C/5'
SH3PX3-R1303	CAAACAKCTCYCCGATGTTCTC	1st	Li *et al*. 2007			
SH3PX3-F532	GACGTTCCCATGATGGCWAAAAT	2nd	Li *et al*. 2007	94°C/3'	30x 94°C/30", 65°C/45", 72°C/1'30"	72°C/5'
SH3PX3-R1299	CATCTCYCCGATGTTCTCGTA	2nd	Li *et al*. 2007			

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Table S2 Results of log10 (Bayes Factors) where Tree 1 = considering collection sites as terminals, Tree 2 = considering river systems as terminals, Tree 3 = considering MAQ+FOR+MAM as one terminal and ARA as a different terminal, Tree 4 = considering FOR+MAM as one terminal, MAQ as another terminal and ARA as a different terminal (based in three mtDNA tree clades); Tree 5 = considering the system of three drainages as terminals; and Tree 6 = considering all *Cyanocharax itaimbe* populations as a single terminal. Values in bold demonstrate evidence in favour of Tree 4 hypothesis.

	ln P(model | data)	S.E.	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	Tree 6
Tree 1	-5434.995	+/- 0.097	-	-2.427	-1.442	-8.812	-7,486	-1.641
Tree 2	-5429.406	+/- 0.095	2.427	-	0.985	-6.385	-5,059	0.786
Tree 3	-5431.674	+/- 0.074	1.442	-0.985	-	-7.369	-6,043	-0.199
Tree 4	-5414.705	+/- 0.095	**8.812**	**6.385**	**7.369**	**-**	**1,326**	**7.17**
Tree 5	-5417,758	+/- 0,093	7,486	5,059	6,043	-1,326	-	5,845
Tree 6	-5431.216	+/- 0.078	1.641	-0.786	0.199	-7.17	-5,845	-

Appendix S2 Additional figures (Figures S1–S3).

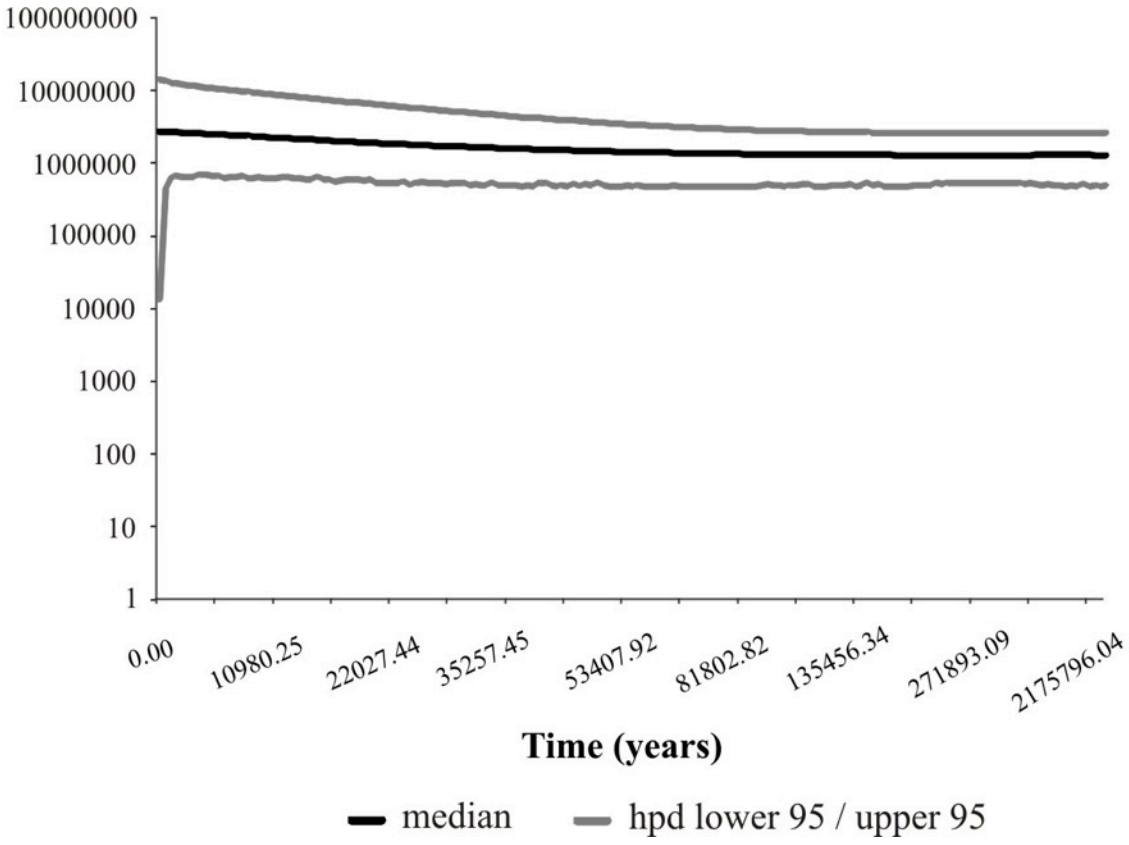

Figure S1 Extended Bayesian Skyline plot with *COI* and *ND2* mitochondrial genes and *SH3PX3* nuclear gene for *Cyanocharax itaimbe* populations in this study.

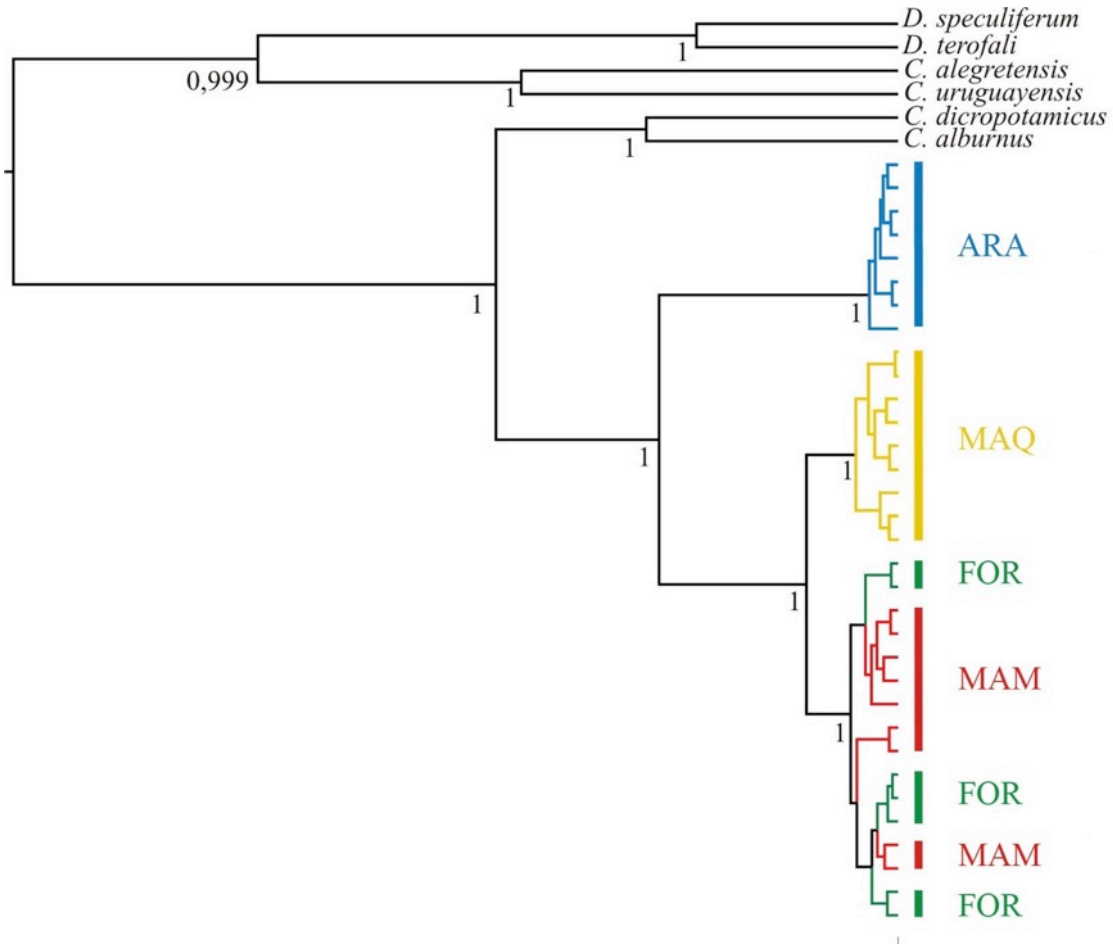

Figure S2 Haplotypic Bayesian tree inferred with mtDNA (*COI* and *ND2* genes). Colored branches indicate the sampled population. Black numbers on the nodes represent posterior probabilities higher than 0.5. ARA = Rio Araranguá; MAM = Rio Mampituba; FOR = Rio Três Forquilhas and MAQ = Rio Maquiné.

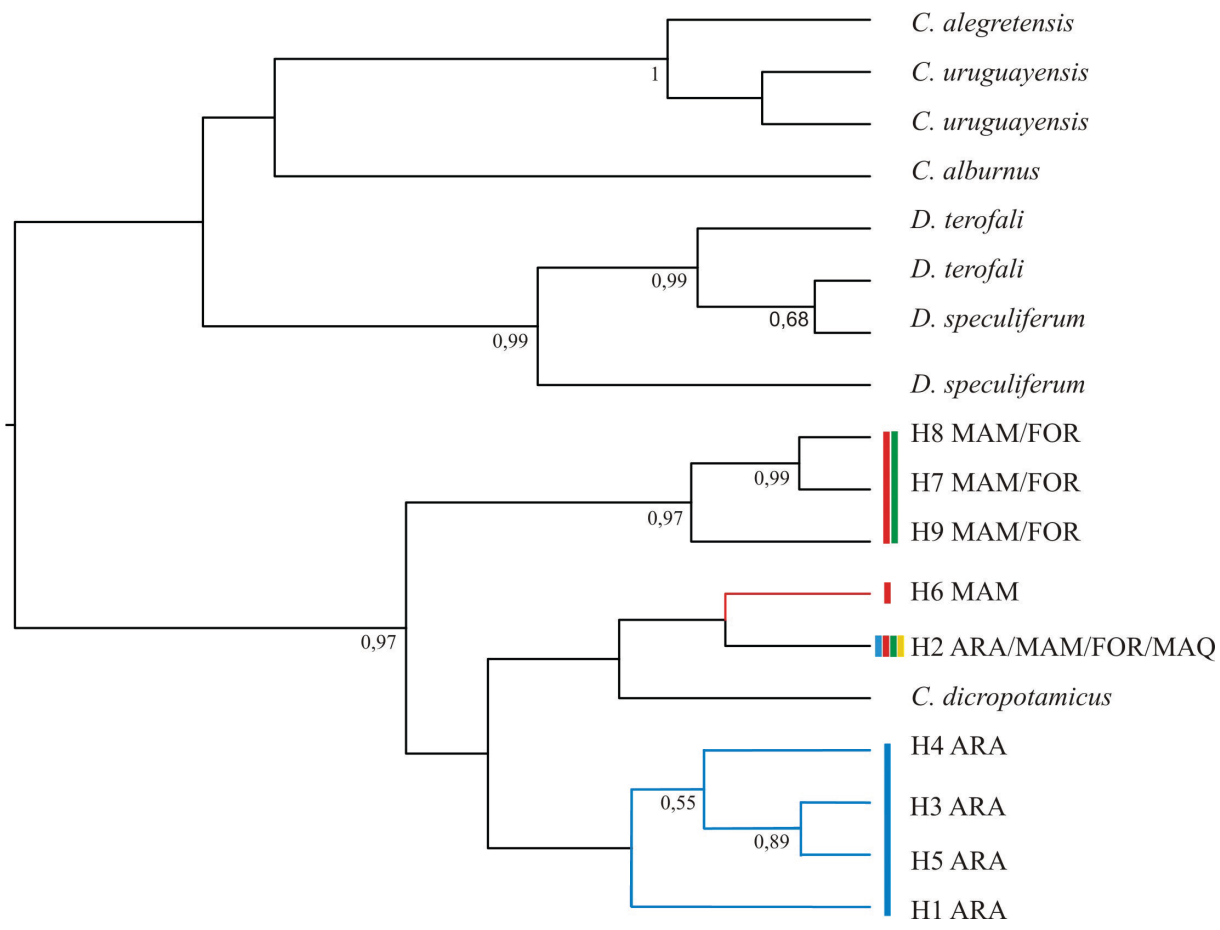

Figure S3 Haplotypic Bayesian tree inferred with the nuclear gene *SH3PX3*. Coloured branches indicate the corresponding population. ARA = Rio Araranguá; MAM = Rio Mampituba; FOR = Rio Três Forquilhas and MAQ = Rio Maquiné.

Capítulo II

Aviso

O Capítulo II segue as normas do periódico Zoological Journal of the Linnean Society.

Redescription and genetic variation of the smiling tetra, *Bryconamericus lethostigmus* (Gomes, 1947) (Characidae: Stevardiinae)

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Running title: Why young *Bryconamericus lethostigmus* doesn´t smile?

Bryconamericus lethostigmus was described as the type species of the monotypic characid genus *Odontostoechus.* Recently a new proposal of classification of the Stevardiinae placed *Odontostoechus* as a junior synonym of a monophyletic genus *Bryconamericus sensu stricto.* However there is an alternative relationship scenario based on primary hypotheses of homology through direct comparisons of the mouth morphology. We redescribe *B. lethostigmus* and analysed the mouth shape and the origin of a single tooth series in the premaxilla in a species of a group characterized by the presence of two tooth series. Moreover we analyse the genetic variation found in the populations to test if this species presents the same phylogeographic pattern observed on the sympatric species *Diapoma itaimbe.* We also test the hypothesis of occurrence of a new species to Rio Araranguá drainage. We analysed 45 specimens characterized for two mitochondrial genes and 319 specimens were analysed morphologically. This study corroborates the hypothesis that the single tooth row in the *B. lethostigmus* is originated with the merging of the external tooth row with the inner row during ontogeny. We also disagree with the primary hypothesis of homology between the mouth modifications found in *B. lethostigmus* and the genus *Monotocheirodon*. Molecular analyses indicated that the phylogeographic pattern of *D. itaimbe* is not repeated in the phylogeography of *B. lethostigmus*. The results from the morphological and molecular

comparisons among drainages refute the hypothesis of a new species to Rio Araranguá drainage.

ADDITIONAL KEYWORDS: Atlantic Forest – Lambari – Neotropical Fishes – Phylogeography.

INTRODUCTION

Bryconamericus lethostigmus (Gomes, 1947) was described as the type species of the monotypic characid genus *Odontostoechus* Gomes, 1947, originally assigned to the subfamily Cheirodontinae due to the presence of one tooth series in the premaxilla. Gomes (1947) proposed the genus *Odontostoechus* as related to *Distoechus* (= *Deuterodon*), *Othonocheirodus* and *Monotocheirodon* based on the structure of the mouth, and to the last two genera by the presence of two rows of gill rakers on the lower ramus of the branchial arches. Indeed, Böhlke (1954) suggested a new Cheirodontinae tribe, the Monotocheirodontini, composed by *Odontostoechus lethostigmus*, *Othonocheirodus* and *Monotocheirodon*, based on mouth similarities, while Géry (1960; 1977) placed *Odontostoechus* as a junior synonym of *Othonocheirodus*. However, Malabarba (1998) considered *Odontostoechus* as a possibly valid genus encompassing several *Bryconamericus*-like species. This author suggested that the single tooth series in *Odontostoechus* originates during premaxilla ontogeny from the merging of the two tooth series, and therefore, that the presence a single tooth series is not homologous to other Cheirodontinae species, placing the genus as *incertae sedis* in Characidae.

Malabarba & Weitzman (2003) provided a better resolution for the phylogenetic relationships among the species described by Gomes by diagnosing a monophyletic group of characid fishes, referred therein as Clade A that included *Odontostoechus lethostigmus*. This hypothesis was corroborated by molecular (Javonillo *et al*. 2010; Oliveira *et al*. 2011) and morphological (Mirande 2010) phylogenies that found Clade A monophyletic. Mirande (2010) named that clade Stevardiinae, which was further corroborated by Baicere-Silva *et al*. (2011), who found that the spermiogenesis process in *Odontostoechus lethostigmus* is homologous in regard to other Stevardiinae.

Among stevardiines, *Odontostoechus* has been hypothesized be more closely related to *Creagrutus*, *Nantis*, and *Piabina* (Mirande, 2010) based on an implied weighting parsimony analysis including several characters. However, Menezes *et al*. (2013) proposed, likewise Böhlke (1954), that *Odontostoechus* was closely related to *Bryconacidnus*, *Ceratobranchia*, *Monotocheirodon*, *Otonocheirodus* and *Rhinopetitia* or to these genera plus *Rhinobrycon* (Netto-Ferreira *et al*., 2014), based on similarities in the mouth shape. On the other hand, the molecular phylogenies of Javonillo *et al*. (2010) and Oliveira *et al*. (2011) grouped *Odontostoechus* with *Bryconamericus* and *Hypobrycon,* and more recently, Thomaz *et al*. (2015a) proposed a new classification for Stevardiinae tribes and genera, placing *Odontostoechus* as a junior synonym of a monophyletic genus *Bryconamericus sensu stricto*.

Bryconamericus lethostigmus is a specialized riverine fish from South Brazilian coastal drainages, originally described from the Rio Maquiné (Rio Tramandaí drainage). Morphologically similar populations have been found on the northern neighbour drainages of Rio Três Forquilhas (also from Rio Tramandaí drainage), Rio Mampituba and Rio Araranguá. Malabarba (1998) suggests that the population from the Rio Araranguá may represent a new species. These river drainages comprise the Tramandaí-Mampituba Freshwater Ecoregion (unit 335 - Abell *et al*., 2008; Fig. 1), an area of high endemism of fish species because of the congruent distributional pattern among several species that are solely found among these three drainages (Malabarba & Isaia, 1992; Reis & Schaefer, 1998). Recently, Hirschmann *et al*. (2015) showed that *Diapoma itaimbe* (Malabarba & Weitzman, 2003), another member of Stevardiinae, has a very strong genetic structure among these drainages, and that the Rio Araranguá population is the most distinct among them. *Bryconamericus lethostigmus* and *D. itaimbe* are sympatric and share the same habitats with clear and cold waters over rocky substrates, leading to the hypothesis that *B. lethostigmus* should also have strong population structure among these drainages.

In this study, we redescribe *Bryconamericus lethostigmus* based on the examination of several populations including that of Rio Maquiné from where the species was originally described (Gomes, 1947). Next, we analyse the genetic variation found in the populations of *B. lethostigmus* from the Tramandaí-Mampituba ecoregion (Abell *et al*., 2008) to test if this species presents the same phylogeographic structure observed for *D. itaimbe* (Hirschmann *et al*., 2015), and if the Rio Araranguá population belongs to a different species (Malabarba, 1998). Finally, we describe and analyse the mouth shape and the origin of a single tooth series

in the premaxilla in a species of a group characterized by the presence of two tooth series, and discuss its phylogenetic implications and possible homologies with other stevardiines.

MATERIAL AND METHODS

Morphological analyses

Measurements and counts were taken following Fink & Weitzman (1974) from specimens belonging to all four river drainages inhabited by this species (Rio Araranguá – ARA; Rio Mampituba – MAM; Rio Três Forquilhas – FOR; and Rio Maquiné – MAQ, which we consider distinct populations). Counts of vertebrae, teeth and procurrent caudal-fin rays were taken from cleared and stained specimens (c&s) prepared according to Taylor & Van Dyke (1985). We included the four vertebrae of the Weberian apparatus in vertebral counts, and counted the terminal centrum as a single element. We took measurements point to point with a calliper on the left side of specimens. We recorded all measurements as percents of standard length (SL) except for subunits of the head, which we recorded as percents of head length (HL). Sex of adult specimens of *Bryconamericus* was determined by the presence (males) or absence (females) of bony hooks in fin rays. Differences in measurements and counts among the four populations were tested with the Kruskal-Wallis test and post-test multiple comparisons using pgirmess package (Giraudoux, 2013) in R (R Core Team, 2013). We used the software Past (Hammer *et al*., 2001) to perform Principal Component Analysis (PCA) with morphometric data. All measurements were log-transformed to compare morphometric variations between populations. Specimens examined belong to the following institutions: MCP (Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil), UFRGS (Departamento de Zoologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil), UMMZ (University of Michigan, Museum of Zoology, Ann Arbor, Michigan, USA) and NMNH (National Museum of Natural History, Smithsonian Institution, Washington D.C., USA).

Molecular analyses

We used tissue samples from 45 specimens of *Bryconamericus lethostigmus* collected throughout its distribution and maintained in 96% ethanol in the fish collection at Departmento de Zoologia, Universidade Federal do Rio Grande do Sul (UFRGS). Samples include individuals from all four river systems in the range of this species (Rio Araranguá –

ARA; Rio Mampituba – MAM; Rio Três Forquilhas – FOR; and Rio Maquiné – MAQ) (Table 1).

DNA extraction followed a modified salt precipitation protocol (Medrano *et al*., 1990). For each sample we used PCR to amplify two mitochondrial genes: cytochrome oxidase subunit I (*COI*) and the NADH dehydrogenase 2 (*ND2*) and three nuclear genes: SH3 and PX domain-containing 3-like protein (*SH3PX3*), S7 ribosomal protein intron 2 (*S72*) (Cooke & Beheregaray, 2007) and myosin heavy chain 6 gene (*Myh6*) (Li *et al*., 2007). PCRs were carried out in 20µl reactions containing 10-50ng DNA, 0,2µM of each primer, 0,2mM of each dNTP, 1x Buffer, 1,5µM $MgCl_2$ and 1U Platinum Taq DNA polymerase (Invitrogen, São Paulo, BR). PCR conditions and primers are presented in Table S1 (Appendix S1). PCR products were checked by electrophoresis in agarose gel, purified using EXOSAP (Exonuclease I and Shrimp Alkaline Phosphatase GE Healthcare®, Piscataway, USA) and sequenced in both directions in Macrogen Inc, Seoul, South Korea.

Forward and reverse reads were assembled and visualized using Geneious 5.6.7 (Drummond *et al*., 2012). The consensus sequences were automatically aligned using the software CLUSTALW (Thompson *et al*., 1994) in BIOEDIT 7.1.3.0 (Hall, 1999) with default parameters. The mitochondrial coding genes *COI* and *ND2* were concatenated for all analyses. Basic descriptive statistics, such as nucleotide (π) and haplotype diversity (hd) as well as neutrality tests were calculated in the software DNASPv5 (Librado & Rozas, 2009). Genetic distances among populations were calculated using the Kimura 2-parameter (K2P) substitution model (Kimura 1980) using MEGA 5 (Tamura *et al*., 2011). Genetic structure among populations was quantified through the Analysis of Molecular Variance (AMOVA) carried out in the program ARLEQUIN 3.5 (Excoffier *et al*., 2005). For this analysis, we considered individuals sampled in each river drainage as coming from different populations.

Evolutionary relationship among haplotypes were estimated based on the median-joining method (MJN) (Bandelt *et al*., 1999) using the program NETWORK 4.1.0.8 (www.fluxus-engineering.com). Estimates the mtDNA coalescence time and the population effective size (Ne) using the BEAST 1.8.2 package (Drummond *et al*., 2012). The mtDNA data set was analysed assuming an evolutionary rate of 0.01/site/Myr (Bermingham *et al*. 1997; Reeves & Bermingham 2006; Ornelas-García *et al*. 2008) and a strict clock model, which is a generally well justified for analysis within a species or among a few closely related species (Li & Drummond 2012). To compare the evolutionary history of *B. lethostigmus* to that of a sympatric species, we estimated coalescence times and population sizes for *Diapoma*

itaimbe based on sequences obtained from GenBank (KP399679 to KP399733; KP406648 to KP406702; Hirschmann *et al*. 2015). Based on the Bayesian Information Criterion in PartitionFinder (Lanfear *et al*., 2012) we assumed the HKY+I substitution model for *COI* and *ND2*. Ten million Markov chain Monte Carlo (MCMC) steps were performed for coalescence time estimation and samples were collected every 1,000 steps. The efficiency of the chain was assessed in Tracer 1.5 (Rambaut & Drummond, 2009) with 10% burn-in.

Examined material. *Odontostoechus lethostigmus* (Gomes, 1947), All from Brazil. Rio **Maquiné basin, type specimens**: UMMZ 143272, 1, 48.68 mm SL, holotype, Rio Grande do Sul, Rio Maquiné, UMMZ 143271, 11, 23.25 - 34.05 mm SL, paratype, Rio Grande do Sul, Rio Maquiné, USNM 143847 [ex UMMZ 143271] 1, 29.97 mm SL, paratype, Rio Grande do Sul, Rio Maquiné. **Rio Maquiné basin, non-type specimens**: UFRGS 3336, 2, 62.65 - 63.15 mm SL, Rio Grande do Sul, Rio Maquiné, under the bridge near Maquiné city, UFRGS 4524, 2, 71.92 - 73.37 mm SL, Rio Grande do Sul, Arroio do Ouro, between Maquiné and Barra do Ouro, UFRGS 4416, 1, 35.97 mm SL, Rio Grande do Sul, Rio Maquiné, UFRGS 4501, 1, 65.44 mm SL, Rio Grande do Sul, Arroio do Ouro, between Maquiné and Barra do Ouro, UFRGS 4377, 2, 35.23 - 37.8 mm SL, Rio Grande do Sul, Rio Maquiné, under the bridge near Maquiné city, UFRGS 4378, 2, 34.37 - 37.72 mm SL, Rio Grande do Sul, Rio Maquiné, under the bridge near Maquiné city, MCP 13657, 4, 30.17 - 36.96 mm SL, Rio Grande do Sul, Maquiné, MCP 14645, 2, 42.67 - 46.76 mm SL, Rio Grande do Sul, Rio Maquiné, MCP 13608, 4, 42.45 - 63.85 mm SL, Rio Grande do Sul, Rio Maquiné, MCP 26965, 5, 22.28 - 68.18 mm SL, Rio Grande do Sul, Maquiné city, MCP 10776, 5, (2 c&s) 40.75 - 55.19 mm SL, Rio Grande do Sul, Rio Maquiné, Maquiné city, MCP 10774, 4 (c&s), 29.77 - 50.43 mm SL Rio Grande do Sul, Maquiné city, Arroio Água Parada. **Rio Três Forquilhas basin**: UFRGS 6309, 2, 55.44 - 67.36 mm SL, Rio Grande do Sul, Rio Três Forquilhas in Vila Boa União, UFRGS 5056, 3, 65.37 - 67.61 mm SL, Rio Grande do Sul, Rio Três Forquilhas in Vila Boa União, UFRGS 2998, 9, 39.33 - 44.14 mm SL, Rio Grande do Sul, Rio Três Forquilhas, UFRGS 6644, 6, 34.53 - 40.9 mm SL, Rio Grande do Sul, Rio Três Forquilhas in Vila Boa União, UFRGS 12736, 2, 23.63 - 45.57 mm SL, Rio Grande do Sul, Rio Três Forquilhas under the high bridge, UFRGS 20710, 2, 23.04 - 58.12 mm SL, Rio Grande do Sul, Rio Três Forquilhas, MCP 25304, 3, 21.63 - 40.39 mm SL, Rio Grande do Sul, Terra de Areia city, MCP 25288, 4, 25.3 - 45.84 mm SL, Rio Grande do Sul, Rio Três Pinheiros, MCP 14314, 6, 32.55 - 47.44 mm SL, Rio Grande do Sul, Rio Três Forquilhas, MCP 25332, 12,

20.67 - 44.95 mm SL, Rio Grande do Sul, Rio Três Forquilhas, MCP 14802, 5, 37.87 - 44.95 mm SL, Rio Grande do Sul, Rio Três Forquilhas, MCP 25673, 8, 33.49 - 45.28 mm SL, Rio Grande do Sul, Rio Três Forquilhas, MCP 21322, 6, 58.25 - 66.04 mm SL, Rio Grande do Sul, Rio Três Forquilhas, MCP 10811, 2 (c&s), 58.95 mm SL, Rio Grande do Sul, Rio Três Forquilhas. **Rio Mampituba basin**: UFRGS 11080, 2, 51.99 - 56.19 mm SL, Santa Catarina, Rio Mampituba, on balneary in Praia Grande, UFRGS 15356, 1, 51.99 mm SL, Rio Grande do Sul, Arroio Paraíso, Morro Azul, UFRGS 16083, 1, 25.99 mm SL, Rio Grande do Sul, Vila São João, Rio Mampituba, UFRGS 19487, 2, 51.63 - 57.03 mm SL, Rio Grande do Sul, Morrinhos do Sul, Arroio Paraíso, UFRGS 19486, 22, (1 c&s) 22.43 - 40.39 mm SL, Rio Grande do Sul, Vila São João, Rio Mampituba, UFRGS 19488, 18, (1 c&s), 20.54 - 47.24 mm SL, Rio Grande do Sul, Torres, Rio Mampituba, UFRGS 20646, 2, 40.12 - 43.18 mm SL, Rio Grande do Sul, Torres, Rio Mampituba, UFRGS 20647, 10, 22.82 - 54.83 mm SL, Rio Grande do Sul, Torres, Rio Mampituba, UFRGS 20648, 1, 55.27 mm SL, Rio Grande do Sul, Morrinhos do Sul, Arroio Paraíso, UFRGS 20649, 3, 23.48 - 41.68 mm SL, Rio Grande do Sul , Vila São João, Rio Mampituba, UFRGS 20650, 2, 35.9 - 46.62 mm SL, Rio Grande do Sul, Morrinhos do Sul, Arroio Paraíso, UFRGS 20652, 2, 46.88 - 49,41 mm SL, Rio Grande do Sul, Morrinhos do Sul, Arroio Paraíso, UFRGS 20653, 4, 49.07 - 58.42 mm SL, Rio Grande do Sul, Arroio Paraíso, UFRGS 20654, 2, 51.8 - 69.06 mm SL, Rio Grande do Sul, Rio Mampituba, UFRGS 20655, 16, 29.06 - 52.34 mm SL, Santa Catarina, Praia Grande, Rio Mampituba, UFRGS 20656, 4, 43.54 - 55.06 mm SL, Rio Grande do Sul, Morrinhos do Sul, Arroio Paraíso, UFRGS 20657, 8, 49.73 - 61.14 mm SL, Santa Catarina, Praia Grande, Rio Mampituba, UFRGS 20658, 5, 30.05 - 45.16 mm SL, Rio Grande do Sul , Vila São João, Rio Mampituba, UFRGS 20659, 2, 32.37 - 43.82 mm SL, Rio Grande do Sul, Morrinhos do Sul, Arroio Paraíso, UFRGS 20660, 12, 42.7 - 65.85 mm SL, Rio Grande do Sul, Morrinhos do Sul, Arroio Paraíso, UFRGS 20708, 4, 28.09 - 41.11 mm SL, Rio Grande do Sul, Vila São João, Rio Mampituba, UFRGS 20709, 6, 38.01 - 45.89 mm SL, Rio Grande do Sul, Rio Mampituba, UFRGS 20711, 2, 59.08 - 60.07 mm SL, Rio Grande do Sul, Morrinhos do Sul, Arroio Paraíso, UFRGS 20712, 3, 49.47 - 61.51 mm SL, Rio Grande do Sul, Morrinhos do Sul, Arroio Paraíso, UFRGS 20713, 1, 47.48 mm SL, Rio Grande do Sul, Rio do Mengue. **Rio Araranguá basin**: UFRGS 10553, 3, 20.97 - 30.74 mm SL, Santa Catarina, Siderópolis, Rio Jordão, UFRGS 15391, 10, 38.26 - 46.3 mm SL, Santa Catarina, Meleiro, Rio Itoupava, UFRGS 15401, 20, (2 c&s) 24.91 - 54.51 mm SL, Santa Catarina, Nova Veneza, Rio São Bento, MCP 23595, 11, 17.48 - 51.3 mm SL, Santa Catarina, Ermo, Rio Itoupava, MCP

19173, 7, (1 c&s), 29.96 - 60.96 mm SL, Santa Catarina, Ermo, Rio Itoupava, MCP 19169, 5, 44.58 - 60.42 mm SL, Santa Catarina, Meleiro, Rio São Francisco, MCP 25436, 2, 25.12 - 66.01 mm SL, Santa Catarina, Ermo, Rio Itoupava, MCP 43602, 1, 50.1 mm SL, Santa Catarina, Ermo, Rio Itoupava. **Rio Urussanga basin**: UFRGS 15385, 1, 39.01 mm SL, Santa Catarina, Urussanga, Rio Cocal.

RESULTS

Bryconamericus lethostigmus (Gomes, 1947)

Fig. 2

Odontostoechus lethostigmus Gomes, 1947: 07 - 12 [original description; holotype: UMMZ 143272; paratypes: CAS-SU 40188 (1) and UMMZ 143271 (originally 12, now 11; 1 specimen posteriorly transferred to USNM 143847); fig. 1 (head and dentition); plate I, fig. 1 (photo of the holotype); type locality: Rio Maquiné, tributary to Lagoa dos Quadros, Conceição do Arroio County, actually Maquiné County, Rio Grande do Sul, Brazil]. - Böhlke, 1954: 25 [listed; *Odontostoechus lethostigmus* as member of the tribe Monotocheirodonini]. - Malabarba, 1998: 204; 231 - 232 [*Odontostoechus* as a valid genus separate from *Othonocheirodus* and *incertae sedis* in Characidae; brief description of the tooth series in the premaxilla; presence of a single tooth series in the premaxilla in *Odontostoechus* hypothesized as non homologous to that of the species of Cheirodontinae]. - Marques *et al*. 2002:28 [categorized as Vulnerable - VU in Rio Grande do Sul state, Brazil, according to IUCN criteria]. Reis *et al*., 2003: 127 [conservation status, distribution, menaces, categorized as Vulnerable - VU in Rio Grande do Sul state, Brazil, according to IUCN criteria]. - Charcansky, 2006: 101 - 102 [tooth morphology and histology], fig. 32 [tooth morphology], fig. 33 [tooth histology]. - Javonillo *et al*., 2010: 505 [phylogenetic relationships]. - Baicere-Silva *et al*., 2011: 379 - 380, 383 [description of spermiogenesis and ultrastructure of the spermatozoa], fig. 12 [scanning electronic images of the ultrastructure of the spermatozoa]. - Oliveira *et al*. 2011 [relationships], 23 [more closely related to *Hypobrycon* and *Bryconamericus exodon*]. - Menezes *et al*. 2013: 143 [possibly related to *Ceratobranchia* cf. *delotaenia*, *Bryconacidnus ellisi*, *Rhinopetitia* cf. *myersi*, *Rhinopetitia* sp., *Otonocheirodus* sp. and *Monotocheirodon*). - Netto-Ferreira *et al*., 2014: 1545 - 1548 [proposal of close

relationships among the genera *Rhinopetitia*, *Bryconacidnus*, *Ceratobranchia*, *Monotocheirodon*, *Odontostoechus*, *Othonocheirodus* and *Rhinobrycon*]. - Malabarba *et al*. 2013: 48 [colour photo, diagnosis, biology, distribution and habitat].
Othonocheirodus lethostigmus. Géry, 1960: 21; 1977: 559 [placed *Odontostoechus* as a junior synonym of *Othonocheirodus*].

Bryconamericus lethostigmus. Thomaz *et al*. 2015a: additional file 5 [*Odontostoechus* as a junior synonym of *Bryconamericus sensu stricto*].

Diagnosis: Bryconamericus lethostigmus is distinct from all other species of the genus by the following autapomorphy: presence of two tooth rows in the premaxilla in small specimens (up to about 30 mm SL) and presence of one tooth row in the premaxilla in large specimens (more than about 40 mm SL) (vs. two tooth rows in the premaxilla regardless body size). This species is distinct from all congeners by the upper lip atrophied in large specimens, leaving the premaxillary teeth exposed.

Description: Morphometric data is summarized in Table 2. Body moderately elongate and compressed. Dorsal profile slightly convex from head until dorsal-fin origin, nearly straight from posterior dorsal-fin base to adipose fin and slightly concave from adipose-fin base to caudal-fin origin. Ventral body profile slightly convex from head to anal-fin origin, straight along anal-fin base and slightly concave from posterior anal-fin base to caudal-fin origin. Greatest depth at dorsal-fin origin or somewhat anterior. Caudal peduncle slightly longer than deep. Dorsal and ventral profiles of caudal peduncle slightly concave.

Mouth large, subterminal, lower jaw shorter than upper jaw and upper lip atrophied (Fig. 3). Snout profile rounded. Premaxilla with two tooth rows in small specimens (up to about 30 mm of SL) and only one tooth row in large specimens (more than about 40 mm SL) (Fig. 4). Single tooth row corresponding to teeth of both rows merged into single series with more teeth than inner or outer series of teeth. Merging of tooth rows gradual; specimens with 30 to 40 mm of SL with well defined double or single series, or in most cases with teeth of inner and outer rows partially merged in single series (Fig. 4). When present, inner row with four teeth with five cusps and outer row with three teeth with three cusps; teeth of inner row pedunculate and wider distally than teeth of outer row. Single premaxillary tooth series with seven, rarely five, six or eigth teeth, equal in size, pedunculate and anteroposteriorly

compressed, with five cusps and sometimes one tooth with six cusps. Maxilla with three to seven teeth with three to five cusps. Last tooth or two posteriormost teeth may be conical in small specimens (Fig. 4). In large specimens two anterior maxillary teeth almost same size of premaxillary teeth and all maxillary teeth exposed (Fig. 4). Dentary with eight to eleven teeth, usually with five cusps, decreasing gradually in size posteriorly; last three teeth very small with fewer cusps; last one or two teeth conical in some specimens (Fig. 5).

Dorsal-fin rays ii, 8 rarely 7, 9 or 10 (mode = 8; n = 318). Dorsal-fin insertion slightly posterior to ventral-fin origin. Adipose fin present. Anal-fin rays iii-v, usually iv or v, 13-19 (mode = 16, n = 317). Pectoral-fin rays i, 9-14 rarely 8 or15 (mode = 12; n = 314). Pelvic-fin rays i, 6-8 rarely 5 or 9 (mode = 7; n = 316). Caudal fin forked, margin of lobes rounded and equal size. Principal caudal-fin rays 19, rarely 17, 18 or 20 (mode = 19; n = 301); 12-14 procurrent caudal-fin rays dorsally (mode = 14; n = 8) and 9-13 ventrally (mode = 12; n = 8).

Scales: cycloid. Lateral line usually complete; number of perforated scales 34-40 (mode = 38, n = 261). One specimen with 29 perforated scales and one with 32. Scale rows between dorsal-fin origin and lateral line 4-6 (mode = 5, n = 307). Scale rows between lateral line and pelvic-fin origin 3-5 (mode = 4, n = 303). Scale rows between lateral line and anal-fin origin 3-4 (mode = 4, n = 301). Predorsal scales 10-14 (mode = 12, n = 314) usually irregularly arranged. Scales sheath along anal-fin base in one row with 1-9 scales (mode = 6, n = 311). Caudal fin not scaled.

Vertebrae: precaudal 16-17 and caudal 19-20 (n = 7). Six vertebrae before first dorsal pterygiophore (n = 8). Supraneurals: 5-6 (n=7).

Statistical results: Some measurements and counts showed significant differences on means among the populations (Table 3), but without any repeatable pattern to distinguish any or a group of populations from all remaining populations. In agreement to the abovementioned results, PCA revealed no differences on measurements of specimens among populations (Fig. 6). Thus, populations showed no morphological significant differences among them.

Color in alcohol: General ground body color yellowish olive. Dorsum dark gray pigmented from head until caudal peduncle. Top of head on frontals and parietals black pigmented, with deep lying black chromatophores over brain membranes under frontals and parietals and fontanel. Ventral region of head light yellowish; cheek and operculum light yellowish with minute black chromatophores concentrated in the upper part of operculum and fifth

infraorbital to form indistinct blotch. Numerous dark gray chromatophores, somewhat contiguous, on snout, upper and lower lips. Body sprinkled with minute black points, most numerous above lateral line, concentrated on posterior margin of the scales. Humeral spot conspicuous above fourth, fifth and part of sixth scale of lateral line. All fins with some black chromatophores along fin rays. Caudal-fin with black stripe. Body with black line along middle longitudinal body axis, beginning above lateral line and reaching caudal-fin stripe.

Color in life: Life color described from a specimen from Rio Tramandaí drainage (Appendix S2). Dorsal portion of head and body light brown. Lateral and ventral portions of head and body white. Humeral spot black and well defined. Midlateral stripe of the body silvery well defined. Iris light red above the pupil. Yellow pigments on dorsal, adipose, caudal, pectoral and pelvic fins and red pigments on anal fin. White pigment on tip of last unbranched and 1st-2nd branched anal-fin rays, and of unbranched and first branched pelvic-fin rays.

Distribution: Bryconamericus lethostigmus is known from the Rio Maquiné and Rio Três Forquilhas (Rio Tramandaí drainage), Rio Mampituba and Rio Araranguá, Atlantic coastal drainages, Rio Grande do Sul and Santa Catarina States, Brazil. There is a collection of a single specimen of *B. lethostigmus* (UFRGS 15385) in the small drainage of the Rio Urussanga, the next Atlantic river drainage northern to the Rio Araranguá.

Sexual dimorphism: Males of *B. lethostigmus* differ from females by having hooks on anal and pelvic fins. Anal-fin rays with tiny bony hooks present on the first 5 to 7 branched rays. Additional tiny hooks are sometimes present in some of remaining branched rays. Hooks usually present on posterior branches and posterior border of lepidotrichia. Usually one hook per ray segment and absent on unbranched ray. Pelvic fin with tiny bony hooks on posteromedial surface of each ray, one hook per segment and absent on unbranched ray.

Ecological notes: Bryconamericus lethostigmus is found in the upper sections of small shallow creeks and rivers draining from Serra Geral formation in Rio Maquiné, Rio Três Forquilhas, Rio Mampituba and Rio Araranguá basins. These rivers have clear and cold waters, rapid flow and a rocky bottom. According to Fontana *et al*. (2003), *B. lethostigmus* diet may be composed of periphyton due to the regression of the upper lip. Stomach contents of three large specimens consisted of a lot of algae (filamentous algae and diatom), some larvae and pupa of Diptera (Psychodidae), larvae of Chironomidae and larvae of Trichoptera.

The presence of these items also indicates a diet composed mostly of periphyton. Stomach contents of four small specimens consisted of highly particulate non-identifiable organic matter (animal or plant origin) with sediment (sand) and presence of filamentous algae and diatom. Vogel (2012) estimated that 58 mm Total Length (TL) is the size at first maturity to *B. lethostigmus* and also that 70 mm TL is the size that all are able to reproduce.

Conservation status: The populations from the Rio Tramandaí and Mampituba drainages were included in the list of endangered species from Rio Grande do Sul State, Brazil, and categorized as Vulnerable - VU, according to IUCN criteria (Marques *et al*., 2002: 28; Reis *et al*., 2003: 127) due to mainly the threat to habitat destruction. In the last one list of endangered species from Rio Grande do Sul State, Brazil, *B. lethostigmus* was not classified in endangered threat categories (Rio Grande do Sul, 2014) due to its population is apparently stable and no eminent threats were identified. The populations from the Rio Araranguá, Rio Urussanga and Mampituba drainages were not included in the list of endangered species from Santa Catarina State (Santa Catarina, 2011). Also this species is not included in the national list of endangered species (MMA, 2014).

We have tried to collect additional specimens of *B. lethostigmus* in the Rio Urussanga and tributaries, the northernmost record of the species, but we were unable to find new specimens in this drainage. This river basin is currently very impacted by coal mining activities, which may affect the occurrence of this species.

Molecular results. A total alignment of 1,700 base pairs (bp) was obtained for the mitochondrial genes (*COI*, 676 bp and *ND2*, 1,024 bp). The nucleotide and haplotype diversity were 0.0029 and 0.98, respectively. A total of 57 polymorphic sites defining 38 different haplotypes for *B. lethostigmus* were found. Neutrality tests were significant, rejected the null hypothesis of constant population size and/or no natural selection (Tajima's D -2.3318, $P<0.01$; Fu's FS -25.65, $P<0.01$). We obtained an alignment of 328 bp for *S72*, 700 bp for *SH3PX3*, and 759 bp for *Myh6*, but all of them were not variable and, therefore, were not used in further analyses. New sequences generated in this study were submitted to GenBank (xxxxx to xxxxx).

Mitochondrial data showed no association between haplotypes and drainages (Fig. 7) and no strong genetic structure among predefined populations. Also there are no haplotypes shared among drainages, except for only two haplotypes shared between FOR and MAM.

Thus, the lack of genetic structure is due to a shallow genealogical structure rather than shared haplotypes. In agreement to the haplotype networks, the AMOVA (Table 4) showed low isolation among river systems for the mtDNA data (Φ_{ST}=0.08). Thus, mitochondrial DNA genetic distance among populations (about 0.3%) was also is very low (Table 5).

The mtDNA coalescence time of *B. lethostigmus* was around 0.34 millions of years ago (Ma) (95% Highest Posterior Density [HPD] 0.15 - 0.58 Ma), more recent than the estimate for *D. itaimbe*, which was around 2 Ma (95% HPD 0.97 - 3.5 Ma). The coalescence time for the MAM+FOR group was very similar for both species 0.32 Ma (95% HPD 0.14 - 0.56 Ma) and 0.31Ma (95% HPD 0.12 - 0.55 Ma) for *B. lethostigmus* and *D. itaimbe* respectively. The Ne also was similar for both species, being 1.2 million (95% HPD 0.55 - 2.23 million) effective females for *B. lethostigmus* and 0.86 million (95% HPD 0.40 - 1.63 million) effective females for *D. itaimbe*.

DISCUSSION

Genetic Structure and Evolutionary History

Both morphological and molecular data refute the hypothesis that the population from the Rio Araranguá possibly constitute a new species (Malabarba, 1998). We have found no clear morphological differences to distinguish the Rio Araranguá or any of the studied populations from all other populations. We found statistical differences in the mean of some measurements or counts in pairwise comparisons, but these differences were not consistent among all populations to allow the diagnosis of any distinct population (Table 3). For example, the mean dorsal-fin height is statistically different between the Rio Araranguá and Rio Três Forquilhas populations, but does not distinguish any of these populations from both the Rio Maquiné and Rio Mampituba populations. The shallow genealogical depth of the mtDNA combined with its lack of geographic structure, and the lack of genetic variation in the nuclear markers are also strong indicators that all *B. lethostigmus* populations constitute a single species.

Considering that *B. lethostigmus* and *D. itaimbe* occur in the same drainages, are syntopic, and share a similar life history, the different phylogeographic pattern found for both species is striking. While *D. itaimbe* shows a strong genetic structure with well-defined mtDNA clades (Hirschmann *et al*., 2015), *B. lethostigmus* showed no genealogical structure,

and only a very weak genetic structure based on mtDNA data. Such phylogeographic difference between these species may be explained by three different scenarios, or a combination of them: (1) Different mtDNA evolutionary rates between the two species; (2) Different colonization times of the two species in these drainages; and (3) Gene flow among *B. lethostigmus* populations through paleaodrainages hampering population structure.

Given the differences on time to most recent common ancestor (TMRCA) for mtDNA, but the similar effective population size estimates, one could propose that *B. lethostigmus* had a mtDNA evolutionary rate ten times slower than *D. itaimbe*. Nabholz *et al*. (2008; 2009) showed that mtDNA evolutionary rate, based on cytochrome b sequence, exhibit a thirty-fold range variation across bird lineages and of hundred-fold variation across mammal lineages. However, such wide evolutionary rate variation was found among distinct taxonomic groups showing differences in generation time and metabolic rate. On the other hand, *B. lethostigmus* and *D. itaimbe* are related species, with similar generation time and metabolic rates. Tringali *et al*. (1999) found only a 0.5-fold difference in mtDNA evolutionary rate for fish species of the same genus. Thus, a difference on evolutionary rate among *B. lethostigmus* and *D. itaimbe* seems an unlikely explanation for their distinct phylogeographic patterns.

Therefore, if these two species have similar mtDNA evolutionary rates, they probably had different evolutionary histories, as illustrated by their different TMRCA for mtDNA lineages. One possibility is that the ancestral of these two species colonized these drainages in different periods. The ancestral of *D. itaimbe* may have arrived in this ecoregion around 2 Ma, while the ancestral of *B. lethostigmus* only arrived around 0.34 Ma, and therefore, the lack of genetic structure among *B. lethostigmus* population may be due, at least in part, to a short evolutionary time. These ancestors may have reached its current distribution by headwater capture or through potential connections on coastal plain during marine regressions, but based on present data it is not possible to state which was the dispersal route followed by these species.

Another possibility for the lack of genetic structure in *B. lethostigmus* is a more intense gene flow among populations over time through palaeodrainages during the marine regressions compared to *D. itaimbe*. The reconstruction of palaeodrainage availability over time (Fig. 8) suggests that these drainages have been isolated only during a short period of time, whereas during most of the time, these drainages had connections, as demonstrated by Thomaz *et al*. (2015b). Under this hypothesis, *B. lethostigmus* would be more efficient in maintaining genetic connectivity among populations than *D. itaimbe*. A problem with this

hypothesis is that if this species had used it these connections until very recently, this would result in a larger amount of shared haplotypes among drainages, which is not observed. There are two mitochondrial haplotypes shared between FOR and MAM populations of *B. lethostigmus*, while the other populations share no haplotypes. This is the same pattern observed for *D. itaimbe*. This common pattern of haplotype sharing observed for both species may be related to a single event that allowed gene flow between these two drainages, most probably headwater capture (Hirschmann *et al*., 2015), a process still active in the rivers of the coastal region (Ribeiro, 2006). According to our results, this event may have occurred around 0.3 Ma, probably close to the dispersal of the ancestral of *B. lethostigmus* across costal drainages.

The hypothesis of headwater capture instead of dispersion through low land connections to explain the sharing haplotypes between the Rio Três Forquilhas and Rio Mampituba may be further supported by the lack of shared haplotypes between FOR and MAQ populations, which are currently connected through freshwater lagoons. The region is widely visited for field works, and there are no capture records for this species in lagoons. Therefore, it is likely lagoons act as barrier to dispersion of *B. lethostigmus* between river systems similarly to what was proposed for *D. itaimbe* (Hirschmann *et al.*, 2015).

Mouth Mophology

The presence of a single tooth series in the premaxilla has been a key character in Eigenmann´s (1915) definition of the characid subfamily Cheirodontinae, that have had up to 56 genera assigned along its history (Malabarba, 1998: appendix B), including *Odontostoechus*. The single tooth series in the premaxilla, however, has been demonstrated by direct homology (Malabarba, 1998) or by parsimony analyses to originate several times and independently in different genera of the Characidae (*e. g. Paracheirodon,* Weitzman & Fink, 1983; *Charax,* Lucena, 1987; "the Rosy Tetras Clade," Weitzman & Palmer, 1997; Xenurobryconini, Weitzman & Fink, 1985; Netto-Ferreira *et al*., 2013).

Malabarba (1998) identified three non-homologous processes of origin of a single tooth series in Characidae: (1) the merging of both rows in a single one; (2) the reduction of the anterior tooth row, with one or even two teeth remaining and not perfectly aligned in a single series; and, (3) the complete loss of the anterior tooth row. According to Malabarba (1998) the single tooth row in the *B. lethostigmus* is originated with the merging of the external tooth row with the inner row during ontogeny and then this character is non-

homologous with the single tooth row observed along all the ontogenetic development in Cheirodontinae. This observation is corroborated in this study where we provided the description and illustration of the merging of the two tooth rows in a single series along ontogenetic development without teeth loss in *B. lethostigmus*.

Among stevardiines, relationships of *B. lethostigmus* have been alternatively assigned to two groups of genera. Phylogenies based on comprehensive samples of characid taxa (Mirande, 2010; Javonillo *et al*., 2010; Oliveira *et al*., 2011; Thomaz *et al*., 2015a) have found the type species of *Odontostoechus* grouped to at least one representative of the genus *Bryconamericus sensu stricto* (Thomaz *et al*., 2015a), including the genera *Hypobrycon* and *Nantis* (currently junior synonyms of *Bryconamericus*). Alternatively, scenarios based on primary hypotheses of homology through direct comparisons of the mouth morphology and others morphological characteres have resulted in completely different hypotheses, grouping *B. lethostigmus* to genera such as *Bryconacidnus*, *Ceratobranchia*, *Monotocheirodon*, *Otonocheirodus*, *Rhinopetitia*, and *Rhinobrycon* (Böhlke, 1954; Menezes *et al*., 2013; Netto-Ferreira *et al*., 2014). However, these hypotheses are based on direct observations and have not been submitted to congruence tests of homology (de Pinna, 1991).

We herein refute the primary hypothesis of homology between mouth modifications found in *B. lethostigmus* and the stevardiine genus *Monotocheirodon*. So far, tooth row reduction in the premaxilla along ontogenetic development observed in *B. lethostigmus* represents a unique condition among the Stevardiinae. The juvenile stage in *B. lethostigmus*, characterized by two premaxillary tooth series, is identical and homologous to the juvenile and adult stages in any *Bryconamericus* species. The adult stage in *B. lethostigmus* instead, characterized by a single tooth series that includes the teeth of both rows merged, is a novelty and an autapomorphy of this species. It can be characterized both by the heterochronic process of peramorphosis related with the addition of one step on the development of the premaxilla as well as by heterotopy by the changes in the spatial distribution of the teeth of the inner and outer series in a final stage of growth (Zelditch & Fink, 1996). We can recognize the final condition in *B. lethostigmus* is the result of a causal sequence of events (merging tooth series) - feature A induces feature B - and it is not a temporal change only.

Although mouth shape in adult *B. lethostigmus* remarkably resembles that of *Monotocheirodon* (Menezes *et al*., 2013: figs. 2, 10, 14) they have different origins. The mouth modifications in *Monotocheirodon* species does not originated from ontogenetic changes in mouth morphology. Instead, the single tooth series in the premaxilla of

Monotocheirodon species apparently originate from the complete loss of the external tooth series, since all the species retain a single series of four teeth that seems to be homologous to the inner premaxillary tooth series found in the stevardiines (Menezes *et al*., 2013, fig. 14, illustrated three premaxillary teeth in *Monotocheirodon kontos*, but the description refers to the presence of four teeth in all specimens).

Other diagnostic character of *B*. *lethostigmus* is the upper lip atrophied. Upper lip atrophied is shared with *Deuterodon stigmaturus*, a species sympatric to *B*. *lethostigmus*, *D*. *rosae*, *Henochilus wheatlandi*, *Psaliodon gymnodontus, Bryconacidnus, Ceratobranchia, Monotocheirodon, Othonocheirodus* and *Rhinopetitia* (Lucena & Lucena, 2002; Netto-Ferreira *et al*., 2014). In *D. stigmaturus*, the upper lip atrophied expose all upper maxilla as in *B. lethostigmus*. Lucena & Lucena (2002) also observed that this character is an independent acquisition on these species. On the other hand Netto-Ferreira *et al*. (2014), although not examined in a cladistic context, suggest that this character plus the morphological similarity of outer series teeth of the premaxilla are strong evidence towards a relationship among the stevardiines *B. lethostigmus*, *Bryconacidnus, Ceratobranchia, Monotocheirodon, Othonocheirodus, Rhinobrycon* and *Rhinopetitia*. We also refute this primary hypothesis of homology due to the differences in the origin of the single tooth series and lip reduction during the ontogenetic development of these species.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Additional table (S1).

Appendix S2 Additional figure (S1).

Table 1 Populations of *Bryconamericus lethostigmus*, voucher specimens and geographical coordinates for the tissues samples used in this study. All samples belong to UFRGS fish collection (Universidade Federal do Rio Grande do Sul).

Population (Drainage)	Voucher	Lat.	Long.
Rio Maquiné / Tramandaí	UFRGS 12086 / TEC1233A	-29.6519400	-50.2094400
Rio Maquiné / Tramandaí	UFRGS 16198 / TEC 2277, 2279	-29.6474400	-50.2172220
Rio Maquiné / Tramandaí	UFRGS 16199 / TEC 2313	-29.5702220	-50.2799160
Rio Maquiné / Tramandaí	UFRGS 16200 / TEC 2314, 2315, 2316, 2318, 2319, 2323	-29.5874166	-50.2703055
Rio Maquiné / Tramandaí	UFRGS 16207/ TEC 2349A	-29.6275277	-50.2419722
Rio Maquiné /Tramandaí	UFRGS 16208 / TEC 2351	-29.6474444	-50.2172222
Rio Três Forquilhas/Tramandaí	UFRGS 16204 / TEC 2331, 2333, 2338	-29.5090000	-50.0916111
Rio Três Forquilhas/Tramandaí	UFRGS 16206 / TEC 2343, 2344, 2345, 2346	-29.5410555	-50.0800000
Rio Três Forquilhas/Tramandaí	UFRGS 16209 / TEC 2357, 2358, 2359, 2360	-29.5090000	-50.0916110
Rio Três Forquilhas/Tramandaí	UFRGS 16210 / TEC 2374	-29.4725278	-50.1194167
Rio Mampituba	UFRGS 12537/ TEC 1239A	-29.2469000	-50.0700000
Rio Mampituba	UFRGS 12723/ TEC 1460A	-29.2491110	-49.8487000
Rio Mampituba	UFRGS 16213/ TEC 2491, 2493, 2494 2495, 2497	-29.2265555	-50.0033050
Rio Mampituba	UFRGS 16226/ TEC 2618, 2620, 2621, 2624	-29.1894444	-49.9033611
Rio Araranguá	UFRGS 16211 / TEC 2375, 2377, 2378, 2379, 2380, 2381	-28.9787500	-49.6736111
Rio Araranguá	UFRGS 16212 / TEC 2427, 2430, 2431, 2436	-28.6097222	-49.5545000

Table 2 Morphometrics of the holotype (UMMZ 143272), twelve paratypes (UMMZ 143271; USNM 143847) and others 306 individuals. Standard length is expressed in mm.

		Paratypes					Rio Maquiné					Rio Três Forquilhas				
	Holotype	**N**	**Min**	**Max**	**Mean**	**SD**	**N**	**Min**	**Max**	**Mean**	**SD**	**N**	**Min**	**Max**	**Mean**	**SD**
Standard length (mm)	48.68	12	23.25	34.50	28.73	-	30	22.28	73.37	45.96	-	68	20.67	67.61	39.98	-
Percents of Standard Length																
Predorsal length	51.01	12	51.01	53.57	52.21	0.84	30	48.97	53.44	50.74	1.12	68	48.25	54.32	51.10	1.27
Prepelvic length	44.36	12	44.36	50.88	47.52	1.61	30	45.11	50.25	47.56	1.24	67	44.93	52.34	47.61	1.32
Prepectoral length	22.99	12	23.50	25.65	24.92	0.72	30	21.72	27.74	24.33	1.68	67	21.08	30.20	24.83	2.11
Preanal length	59.85	12	59.85	64.25	62.08	1.31	30	58.48	66.25	63.16	1.63	68	59.16	66.32	62.72	1.45
Body height	24.43	12	24.43	27.88	26.21	0.94	30	24.37	32.55	29.03	2.25	68	23.61	33.57	28.03	2.34
Caudal peduncule height	8.34	12	8.34	10.20	9.28	0.57	30	9.45	11.87	10.59	0.68	68	8.10	12.21	10.28	0.90
Caudal peduncule length	15.85	12	15.85	18.55	17.39	0.92	30	14.57	21.23	17.67	1.76	68	9.59	20.53	17.14	1.59
Anal-fin base length	19.04	12	21.34	27.14	23.31	1.51	30	16.85	24.97	21.32	1.66	68	17.75	24.65	21.83	1.46
Anal-fin height	18.18	12	18.65	22.41	20.60	1.03	30	15.50	21.98	18.84	1.75	68	16.29	24.34	19.57	1.71
Dorsal-fin base length	11.26	12	11.26	14.49	12.48	1.11	30	10.82	14.86	12.72	1.34	68	9.92	19.40	12.88	1.48
Dorsal-fin height	21.16	12	21.33	26.86	24.60	1.78	24	19.86	26.51	24.27	1.71	48	21.79	34.14	24.84	1.78
Pelvic-fin length	-	-	-	-	-	-	30	1.99	4.05	2.94	0.53	68	2.19	4.75	3.01	0.59
Pelvic fin height	13.66	12	13.79	16.99	15.38	0.99	26	12.97	16.44	15.14	0.79	48	11.71	16.69	14.91	1.10
Pectoral-fin length	-	-	-	-	-	-	30	2.96	5.08	3.80	0.57	68	2.65	5.65	3.76	0.64
Pectoral-fin height	17.36	12	19.21	25.29	21.93	1.48	24	16.16	22.14	20.00	1.49	47	16.17	23.29	20.06	1.61
Head length	23.15	11	24.09	27.11	25.89	0.93	30	22.16	27.04	25.01	1.27	68	22.30	29.79	25.47	1.75
Percents of Head Length																
Snout length	22.10	12	22.10	29.12	25.91	1.85	30	24.48	31.56	27.95	1.76	68	22.11	36.42	26.82	2.66
Maxillary length	-	-	-	-	-	-	30	30.79	43.37	38.08	2.47	68	31.50	41.47	37.09	2.42

Table 2 (continued)

		Paratypes					Rio Maquiné					Rio Três Forquilhas				
	Holotype	**N**	**Min**	**Max**	**Mean**	**SD**	**N**	**Min**	**Max**	**Mean**	**SD**	**N**	**Min**	**Max**	**Mean**	**SD**
Premaxillary length	13.83	12	13.83	20.98	17.41	2.27	30	20.27	30.78	25.60	2.63	68	16.16	32.51	23.39	3.67
Eye diameter	39.25	12	39.25	47.86	43.55	2.56	30	29.37	46.42	38.80	3.50	68	21.10	50.97	40.77	3.98
Interorbital	21.14	12	21.14	30.45	27.42	2.82	30	26.06	38.07	30.83	2.62	68	20.82	38.49	29.71	3.48
Mouth width	-	-	-	-	-	-	30	24.15	34.69	28.55	2.55	68	21.78	37.42	28.65	3.10
Counts																
Anal-fin branched rays	15	12	14	16	15.17	0.72	29	13	18	15.72	1.19	68	14	19	16.01	0.98
Dorsal-fin branched rays	8	12	8	8	8.00	0.00	30	7	10	8.00	0.45	68	7	8	7.97	0.17
Pelvic-fin branched rays	7	12	7	8	7.08	0.29	30	6	8	6.93	0.37	68	6	8	6.99	0.32
Pectoral-fin branched rays	10	9	9	12	10.67	0.87	30	11	13	11.90	0.71	68	11	13	12.01	0.44
Caudal-fin branched rays	19	11	18	19	18.73	0.47	22	18	19	18.95	0.21	68	18	19	18.96	0.21
Lateral line scales	37	12	35	38	37.50	0.90	27	36	40	37.93	1.07	60	35	40	37.83	0.87
Rows of scales lateral line/dorsal fin	5	12	4	5	4.92	0.29	28	4	5	4.96	0.19	65	4	5	4.95	0.21
Rows of scales lateral line/pelvic fin	3	12	3	4	3.58	0.51	28	3	5	3.86	0.59	65	3	5	3.89	0.36
Rows of scales lateral line/anal fin	4	12	3	4	3.75	0.45	28	3	4	3.82	0.39	66	3	4	3.91	0.29
Predorsal scales	13	12	10	13	11.33	0.98	30	11	13	11.83	0.70	68	10	14	12.10	0.74
Circunpeduncular scales	14	12	14	14	14.00	0.00	28	13	14	13.93	0.26	54	13	14	13.96	0.19
Scale sheath on anal-fin base	5	12	5	7	6.42	0.79	29	4	7	5.83	0.97	68	2	7	5.57	1.23

Table 2 (continued)

	Rio Mampituba					Rio Araranguá				
	N	**Min**	**Max**	**Mean**	**SD**	**N**	**Min**	**Max**	**Mean**	**SD**
Standard length (mm)	149	20.54	69.06	39.62	-	59	17.48	66.01	38.93	-
Percents of Standard Length										
Predorsal length	149	47.94	56.47	51.43	1.38	59	34.33	56.85	50.77	2.56
Prepelvic length	149	44.67	51.99	47.79	1.61	59	45.17	50.45	47.80	1.19
Prepectoral length	149	21.64	30.45	25.00	2.18	59	22.52	30.78	25.60	1.77
Preanal length	149	57.89	66.88	62.15	1.87	59	57.54	66.49	62.57	1.85
Body height	149	23.15	34.71	28.21	2.20	59	20.48	32.84	27.89	2.77
Caudal peduncule height	149	8.23	16.27	10.60	0.90	59	7.32	12.09	10.38	1.02
Caudal peduncule length	149	11.78	20.84	17.31	1.25	59	13.97	20.28	17.55	1.45
Anal-fin base length	149	19.02	29.08	22.40	1.52	59	17.52	27.27	21.81	1.55
Anal-fin height	149	13.21	25.39	19.92	1.75	59	17.22	24.64	19.82	1.51
Dorsal-fin base length	149	8.80	17.40	12.81	1.32	59	8.44	16.20	12.65	1.42
Dorsal-fin height	67	21.29	40.16	25.43	2.57	10	22.60	27.29	24.57	1.47
Pelvic-fin length	148	2.14	4.98	3.24	0.60	59	1.66	3.81	2.74	0.39
Pelvic fin height	67	12.88	22.19	15.57	1.35	11	14.71	17.03	15.64	0.82
Pectoral-fin length	149	2.85	5.38	4.03	0.54	59	2.49	4.78	3.50	0.44
Pectoral-fin height	66	15.81	27.59	20.51	1.69	11	18.71	23.24	20.78	1.44
Head length	149	22.50	28.65	25.50	1.54	59	22.99	29.57	25.57	1.21
Percents of Head Length										
Snout length	149	20.21	35.87	25.83	2.24	59	21.45	29.79	25.90	1.81
Maxillary length	149	27.07	42.02	36.54	2.22	59	29.65	40.03	35.77	1.99

Table 2 (continued)

	Rio Mampituba					Rio Araranguá				
	N	**Min**	**Max**	**Mean**	**SD**	**N**	**Min**	**Max**	**Mean**	**SD**
Premaxillary length	142	15.55	30.14	22.60	3.38	59	15.28	28.92	21.06	2.70
Eye diameter	149	32.92	49.77	41.06	3.10	59	32.90	50.23	41.39	3.10
Interorbital	149	22.84	48.19	31.13	3.21	59	19.05	33.30	28.87	3.09
Mouth width	149	20.00	36.66	27.81	3.46	58	20.65	33.25	26.45	3.04
Counts										
Anal-fin branched rays	149	13	18	15.74	0.95	58	14	18	16.19	0.76
Dorsal-fin branched rays	149	7	9	7.99	0.14	58	7	8	7.98	0.13
Pelvic-fin branched rays	148	6	8	7.02	0.22	57	6	8	6.93	0.32
Pectoral-fin branched rays	148	10	14	12.14	0.75	58	10	13	11.71	0.62
Caudal-fin branched rays	146	17	20	18.99	0.22	53	17	20	18.96	0.34
Lateral line scales	115	36	40	37.97	0.72	46	34	39	37.63	1.12
Rows of scales lateral line/dorsal fin	147	4	6	4.99	0.16	54	5	6	5.02	0.14
Rows of scales lateral line/pelvic fin	143	3	5	3.80	0.44	54	3	4	3.94	0.23
Rows of scales lateral line/anal fin	141	3	4	3.89	0.31	53	4	4	4.00	0.00
Predorsal scales	148	10	14	12.05	0.77	55	11	14	12.16	0.81
Circunpeduncular scales	116	12	14	13.96	0.24	48	14	14	14.00	0.00
Scale sheath on anal-fin base	146	2	8	5.53	1.10	55	1	9	5.55	1.50

Table 3 Results non-parametric significance test Kruskal Wallis from measures and counts that showed significant differences on averages among the *Bryconamericus lethostigmus* populations. The *B*. *lethostigmus* populations are represented as: ARA = Rio Araranguá; MAM = Rio Mampituba; FOR = Rio Três Forquilhas and MAQ = Rio Maquiné.

Measures and counts	Kruskal-Wallis chi-squared	p-value	Different populations
Dorsal-fin height	8.9028	0.03061	ARA x FOR
Pelvic-fin height	10.478	0.01491	ARA x FOR
Pectoral-fin length	10.41	0.01538	ARA x MAQ
Pectoral-fin height	9.6833	0.02146	ARA x FOR
Maxillary length	8.4277	0.03795	ARA x MAQ / MAM x MAQ
Anal-fin rays	15.46	0.001463	ARA x MAM / ARA x MAQ
Pectoral-fin rays	23.133	3.79E-05	ARA x MAM / MAM x MAQ
Caudal-fin rays	29.916	1.44E-06	MAM x MAQ

Table 4 Results from the analysis of molecular variance (AMOVA) among and within *Bryconamericus lethostigmus* populations for the mtDNA data.

Source of variation	mtDNA		
	d.f	% variation	p-value
Among	3	8.35	< 0.001
Within	41	91.65	< 0.001
Fixation Index Φ		0.08347	< 0.001

Table 5 Genetic distance matrix for mitochondrial data using the Kimura 2-parameter (K2P) among *Bryconamericus lethostigmus* populations. The *B*. *lethostigmus* populations are represented as: ARA = Rio Araranguá; MAM = Rio Mampituba; FOR = Rio Três Forquilhas and MAQ = Rio Maquiné.

	ARA	MAM	FOR	MAQ
ARA	0			
MAM	0.003	0		
FOR	0.003	0.003	0	
MAQ	0.003	0.003	0.003	0

Figure 1 Geographical distribution of *Bryconamericus lethostigmus*. Tramandaí-Mampituba Freshwater Ecoregion; squares represent sampled localities of *B. lethostigmus*.

Figure 2 *Bryconamericus lethostigmus,* holotype, 48.68 mm SL (UMMZ 143272).

Figure 3 Detail of shape mouth with upper lip atrophied of *Bryconamericus lethostigmus,* large specimen, 54.33 mm SL (UFRGS 20660) and small specimen, 24.55 mm SL (UFRGS 16083).

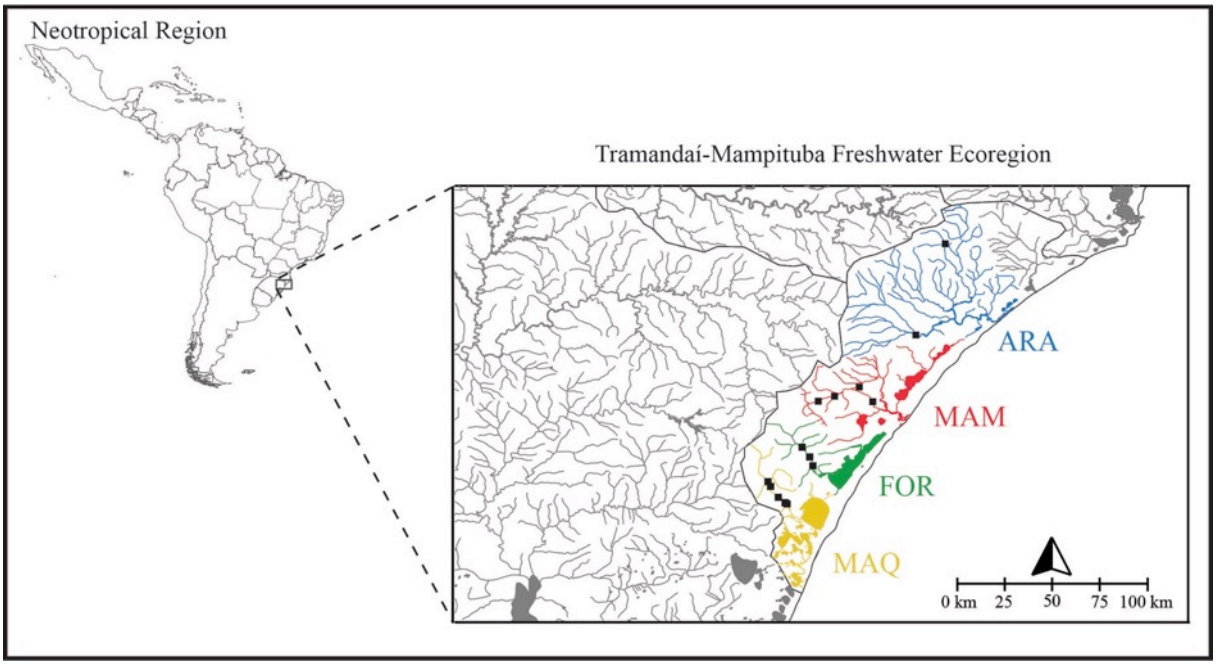
Figure 4 Process of premaxillary tooth row reduction of *Bryconamericus lethostigmus*: (a) specimen with two series of teeth, 24.15 mm SL (UFRGS 19488); (b) specimens are in transition process, is possible identify teeth inner and outer row, 32.44 mm SL and 38.93 mm SL (UFRGS 15401); (c) specimens show a single teeth series, 41.16 mm SL and 50.43 mm SL (MCP10774), 60.96 mm SL (MCP 19173).

Figure 5 Lower jaw of *Bryconamericus lethostigmus*, 29.77 mm SL (MCP 10774), 40.39 mm SL (UFRGS 19486) and 60.96 mm SL (MCP 19173) respectively from left to right.

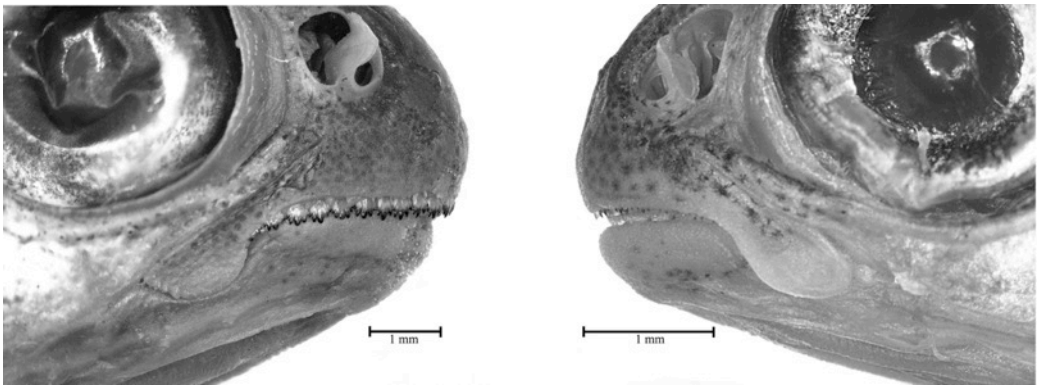
Figure 6 Principal Component Analysis on measurements log-transformed of the *Bryconamericus lethostigmus* populations. Blue square = ARA (Rio Araranguá); red cross = MAM (Rio Mampituba); green cross = FOR (Rio Três Forquilhas) and black circle = MAQ (Rio Maquiné).

Figure 7 Median-joining networks among haplotypes of *Bryconamericus lethostigmus* samples inferred by the concatenated mtDNA dataset (*COI+ND2*). Each circle represents a unique haplotype with circle sizes being proportional to their frequencies. Each colour represents a population. Crossed markers indicate the number of mutations between haplotypes.

Figure 8 Variation in sea level for the last 500 ka shown according to Miller *et al*. (2011).


Neotropical Region
Tramandaí-Mampituba Freshwater Ecoregion
ARA
MAM
FOR
MAQ
0 km 25 50 75 100 km


1 cm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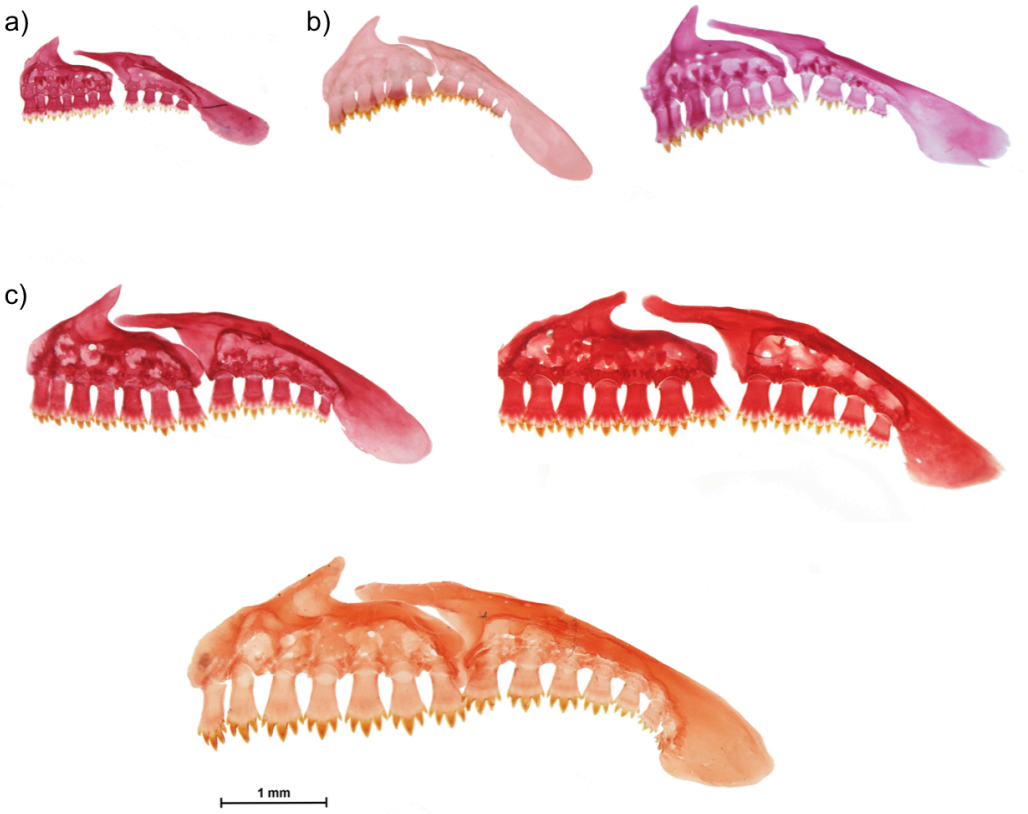
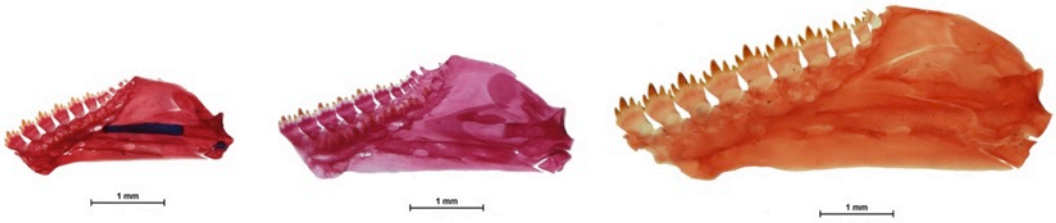
1 mm


a)
b)
c)
1 mm


1 mm
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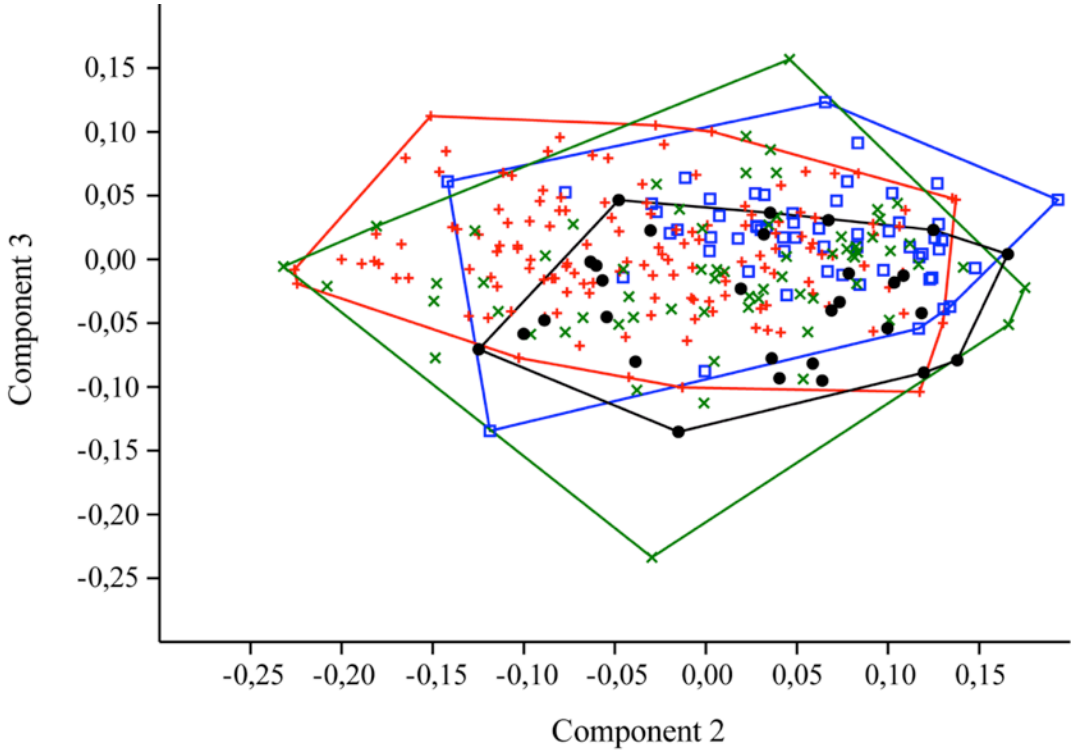
1 mm


0,15
0,10
0,05
0,00
-0,05
-0,10
-0,15
-0,20
-0,25
Component 3
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-0,20
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-0,10
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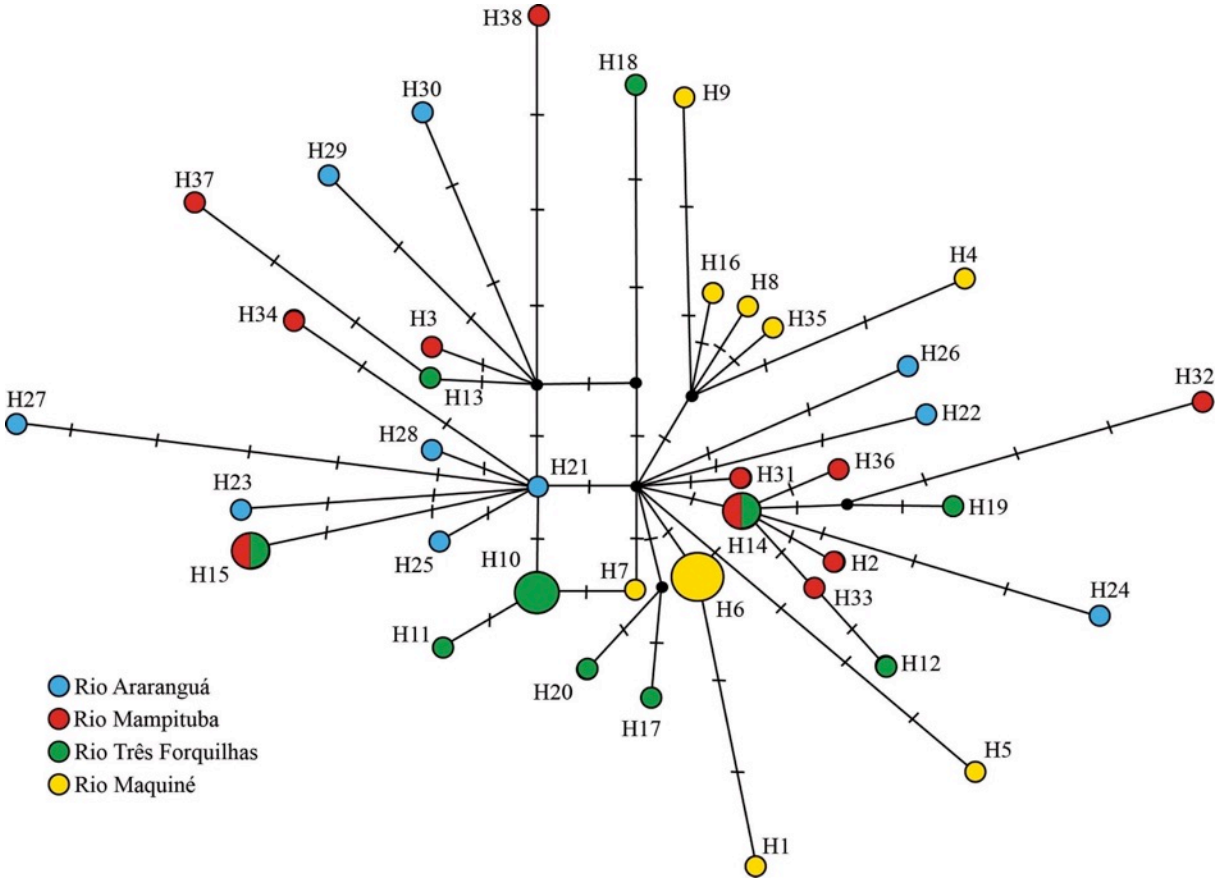
0,15
Component 2


H38
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H9
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H37
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H22
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H36
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H19
H14
H15
H25
H10
H7
H2
H24
H33
H6
H11
H12
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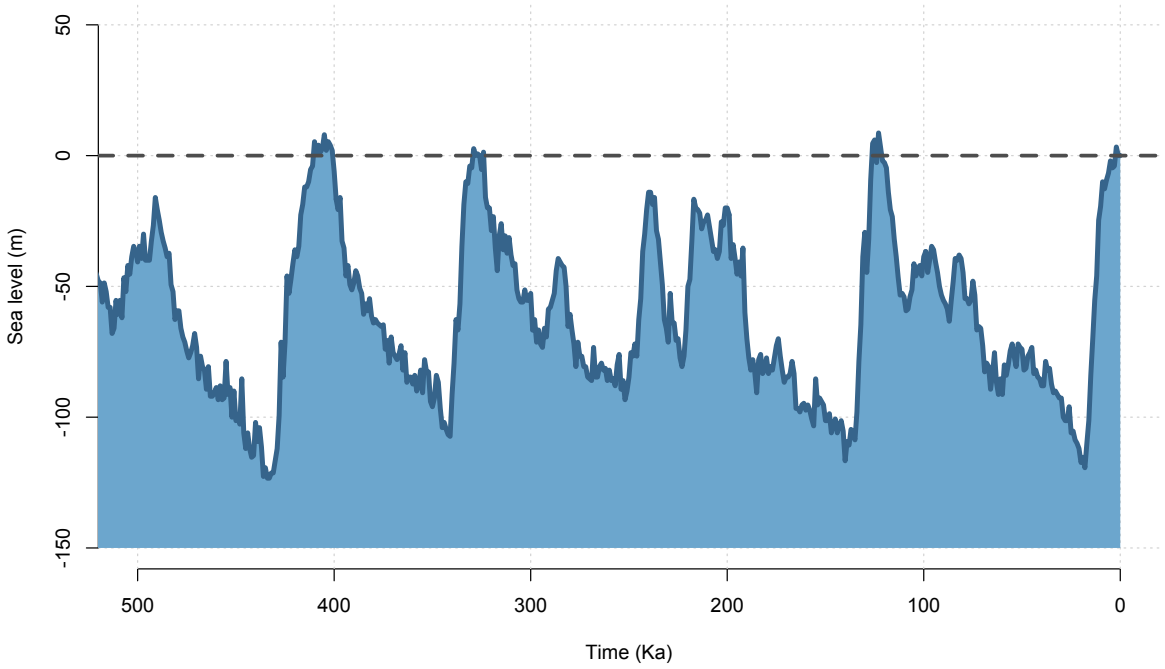
H17
H5
H1
Rio Araranguá
Rio Mampituba
Rio Três Forquilhas
Rio Maquiné


Sea level (m)
50
0
-50
-100
-150
500
400
300
200
100
0
Time (Ka)

Supporting Information

Appendix S1 Additional table (S1)

Table S1 Primers used in this study, with their sequence, references and PCR conditions.

Gene	Primer sequence (liste from 5' to 3')	Reference	Desnaturation	Cycles	Extension
COI-H2198	TAA AcT TcA ggg TgA ccA AAA AAT cA	Herbert *et al*. (2003)		40x 94°C/30", 50°C/20", 48°C/5",	
			96°C/1'	46°C/5", 44°C/5", 42°C/5",	72°C/3'
COI-L1490	ggT cAA cAA ATc ATA AAg ATA TTg g	Herbert *et al*. (2003)		40°C/20", 72°C/1'	
ND2-L5216	GGC CCA TAC CCC GRA AAT G	Sorenson *et al*. (1999)		9x 94°C/30", 57°C-1°C/cycle/1',	
			94°C/4'	72°C/1'30", 40x 94°C/30",	72°C/5'
ND2-H6313	ACT CTT RTT TAA GGC TTT GAA GGC	Sorenson *et al*. (1999)		47°C/1', 72°C/1'30"	

References

Herbert, P. D. N., Cywinska, A., Ball, S. L. & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B*, 270, 313 - 321.

Sorenson, M. D., Ast, J. C., Dimcheff, D. E., Yuri, T. & Mindell, D. P. (1999). Primers for a PCR-Based Approach to Mitochondrial Genome Sequencing in Birds and Other Vertebrates. *Molecular Phylogenetics and Evolution*, 12, 105 - 114.

Appendix S2 Additional figure (S1)

Figure S1 Color in life of the *Bryconamericus lethostigmus*, Rio Três Forquilhas basin, Itati county, Rio Grande do Sul, Brazil, UFRGS 16500 (Photo: Malabarba *et al*. 2013).

References

Malabarba LR, Neto PC, Bertaco VA, Carvalho TP, Santos JF.dos, Artioli LGS. 2013. *Guia de identificação dos peixes da bacia do rio Tramandaí*. Ed. Via Sapiens, Porto Alegre, RS, 140 p.

Capítulo III

Aviso

O Capítulo III segue as normas do periódico Neotropical Ichthyology.

Integrative analysis - Morphological and Molecular Phylogeny of the genus *Diapoma* Cope 1894 (Characidae: Stevardiinae)

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Abstract: The use of morphological and molecular data have been fundamental for the reassessment of the systematics of several Stevardiinae groups. While morphological phylogenies have proposed that *Diapoma* and *Cyanocharax* are distinct genera in Stevardiinae, molecular phylogenies have found that both genera are paraphyletic, even though its species form a single clade. A recent study based on molecular data proposed a new classification for tribes and genera in Stevardiinae, with all species of *Cyanocharax* (together with *Hyphessobrycon guarani*) being reassigned to *Diapoma* in order to define a monophyletic genus consistent with a phylogenetic classification. In this study we try to clarify the phylogenetic relationships among *Diapoma* species through comparative molecular and morphological analyses and simultaneous analysis. Our analyses recovered the monophyly of *Diapoma* (including *Cyanocharax)* irrespective of the dataset used. However, the relationships among *Diapoma* species showed some incongruences across datasets. In general, molecular phylogenies showed higher support for clades compared to morphological phylogenies. Using both molecular and morphological data resulted in a phylogeny showing good resolution for internal relationships of *Diapoma* species, in which it was possible to trace morphological synapomorphies for several clades.

Resumo: A utilização de dados morfológicos e moleculares tem sido fundamentais para a reavaliação sistemática de vários grupos dentro de Stevardiinae. Enquanto filogenias morfológicas propuseram *Diapoma* e *Cyanocharax* como gêneros distintos em Stevardiinae, filogenias moleculares encontraram ambos os gêneros parafiléticos com suas espécies formando um clado único. Recentemente, um estudo baseado em dados moleculares propôs uma nova classificação para tribos e gêneros em Stevardiinae, onde todas as espécies de *Cyanocharax* (juntamente com *Hyphessobrycon guarani*) foram atribuídas à *Diapoma*, a fim de definir um gênero monofilético consistente com a classificação filogenética. Neste estudo tentamos esclarecer as relações filogenéticas entre as espécies de *Diapoma* através de análises moleculares e morfológicas comparativas e uma análise simultânea. Nossas análises recuperaram o monofiletismo de *Diapoma* (incluindo *Cyanocharax*), independentemente do conjunto de dados utilizado. No entanto, as relações entre as espécies de *Diapoma* mostrou algumas incongruências em todos os conjuntos de dados. Em geral, as filogenias moleculares mostraram um suporte maior para os clados em comparação com as filogenias morfológicas. A análise combinada com dados moleculares e morfológicos resultou em uma filogenia com boa resolução para as relações internas de *Diapoma*, nas quais foi possível traçar sinapomorfias morfológicas em vários clados.

Keywords: Total Evidence, Molecular Phylogeny, Lambari, Neotropical Fishes

INTRODUCTION

The monotypic genus *Diapoma* Cope (1894) was originally defined by the modifications of the elongate opercular apparatus observed in the type species, *Diapoma speculiferum* Cope, 1894. Later, Weitzman & Fink (1985) included the species originally described as *Glandulocauda terofali* Géry, 1964 into *Diapoma*, considering that the structure and scale arrangement of the caudal-fin organ in both species was homologous and diagnostic for the genus. Based on the similarity of the caudal-fin organs observed among *Acrobrycon* Eigenmann & Pearson, 1924, *Diapoma* and *Planaltina* Böhlke, 1954, Weitzman *et al*. (1988) included these three genera in the tribe Diapomini. More recently, Menezes & Weitzman (2011) described two new species of *Diapom*a (*D. pyrrhopteryx* Menezes & Weitzman, 2011 and *D. thauma* Menezes & Weitzman, 2011) and maintained the genus in the tribe Diapomini,

subfamily Stevardiinae, noting that robust phylogenetic studies were still lacking for this group.

The genus *Cyanocharax* Malabarba & Weitzman (2003) was proposed to include six new species (*Cyanocharax alegretensis* Malabarba & Weitzman 2003, *C. dicropotamicus* Malabarba & Weitzman 2003, *C. itaimbe* Malabarba & Weitzman 2003, *C. lepiclastus* Malabarba, Weitzman & Casciotta, 2003, *C. macropinna* Malabarba & Weitzman, 2003, and *C. tipiaia* Malabarba & Weitzman 2003) together with a new combination for *C. alburnus* (Hensel, 1870). Although there were no exclusive characters or synapomorphies to diagnose the genus, the genus was considered to belong to the characid Clade A (Malabarba & Weitzman, 2003). Later, Malabarba *et al.* (2004) considered *C. macropinna*, the type species of the genus, a junior synonym of *Hyphessobrycon melanopleurus uruguayensis* Messner, 1962, and renamed in a new combination as *C. uruguayensis*. More recently, Casciotta *et al*. (2012) described a new species, *C. obi* Casciotta *et al*., 2012, resulting in eight species in *Cyanocharax*.

The morphological phylogeny of Characidae proposed by Mirande (2010) recognizes *Cyanocharax* and *Diapoma* as distinct genera in Stevardiinae, and suggests that these genera are not closely related to each other. In a further study, Mirande *et al.* (2013) restated the distinctiveness of the two genera, but suggested that *Cyanocharax* was paraphyletic. In contrast, studies based on molecular markers have provided a very different picture. Javonillo *et al*. (2010) found both *Cyanocharax* and *Diapoma* as paraphyletic, but with the species of the two genera forming a single clade, while Casciotta *et al*. (2012) also suggested that *Cyanocharax* was paraphyletic with the inclusion of *Diapoma*. Recently, Thomaz *et al*. (2015) found that the monophyly of *Cyanocharax* could be strongly rejected by topological tests based on the molecular data. In this study, Thomaz *et al*. (2015) proposed a new classification for tribes and genera of Stevardiinae, with all species of *Cyanocharax* and *Hyphessobrycon guarani* Mahnert & Géry (1987) being reassigned to the genus *Diapoma* in order to define a monophyletic genus consistent with a phylogenetic classification. Furthermore, Thomaz *et al.* (2015) propose the number of pelvic-fin rays (i + 6) as a synapomorphy for this clade, differing from the other genera in Stevardiinae (i + 7). However, this character is also shared with the genus *Planaltina*, which was not analyzed in that study.

Likewise most Characidae, the Stevardiinae are morphologically very conserved (Weiss *et al*., 2012 recorded Characidae fossils with essentially modern morphology for the Eocene-Oligocene), which may explain the high number of homoplasies among modern forms hindering the robust inference of phylogenetic relationships based on morphological

characters only (Weitzman & Fink, 1983; Mirande, 2010; Malabarba *et al.*, 2012; Thomaz *et al.*, 2015). Therefore, it is necessary to search for further morphological characters that could illuminate hypotheses of phylogenetic relationships. Molecular data also are not immune from processes that may mislead phylogenetic inference, such as long branch attraction, paralogy, incomplete lineage sorting, horizontal transfer, rate heterogeneity across lineages and sites, base composition bias, codon usage bias, autocorrelation of adjacent sites, and even large-scale adaptive convergence (Rodríguez-Ezpeleta *et al*., 2007; Philippe *et al*., 2011). Thus, the congruence of phylogenies built from independent evidence is important to establish phylogenetic relationships with confidence (Lee & Palci, 2015).

While analyses of morphology alone might retrieve inaccurate trees due to homoplasies, it might still have a positive impact on phylogenetic accuracy when analyzed in combination with other (mainly molecular) data (Lee & Palci, 2015). When used in total evidence analysis, the morphological data may contribute to tree reconstruction by increasing accuracy, helping to resolve nodes for which molecular data have low resolution (Beutel *et al*. 2011) or by interacting with molecular data to retrieve novel clades that would be missed by morphological or molecular data alone (Gatesy *et al*. 2003; Reeder *et al*., 2015). In such integrated analysis, it is still possible to trace the synapomorphies that define clades. In this study, we aim at clarifying the phylogenetic relationships among *Diapoma* species using molecular, morphological, and total evidence analysis.

MATERIAL AND METHODS

Taxon sampling

We examined specimens belonging to the following institutions: Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre (MCP) and Departamento de Zoologia, Universidade Federal do Rio Grande do Sul, Porto Alegre (UFRGS). The taxonomic classification of the Stevardiinae used in the present paper follows Thomaz *et al*. (2015) while that of the remaining Characidae follows Mirande *et al*. (2013). All specimens used for molecular work have tissue samples preserved in ethanol associated with voucher specimens in museum fish collections and were identified to species (or genus) based on diagnostic morphological traits.

Morphological analyses

The phylogenetic analysis was performed including all species of *Diapoma* in the Extended Matrix of Mirande *et al*. (2013) for a total of 15 terminals in the ingroup. With those additions, the final morphological data set had 105 species.

Two sub-datasets of the Stevardiinae matrix were analyzed. The first sub-dataset includes the characters analyzed by Mirande (2010) with the characters modified by Carvalho (2011) and Mirande *et al*. (2013). We added seven discrete characters to the original list of 392 characters, being three characters from Malabarba & Weitzman (2003), one based on Menezes & Weitzman (2011) and three new characters added herein. Thus, a total of 399 characters were analyzed. The complete list of discrete characters is provided in Appendix S1 and the data matrix is presented in Appendix S2. The second sub-dataset was identical to the first except for the addition of fourteen continuous characters and the removal of eleven discrete characters (125, 135, 136, 138, 139, 286-289, 379, 380) to avoid redundancy with the continuous characters. Thus, a total of 402 characters were analyzed. The complete list and data matrix of continuous characters is provided in in Appendix S3. Data were extracted from the literature (Malabarba & Weitzman, 2003, Menezes & Weitzman, 2011 and Casciotta *et al*., 2012) and from direct examination of specimens (see Comparative Material). Osteological preparations (c&s) were made according to Taylor & Van Dyke (1985).

Continuous characters were treated without discretization as described in Goloboff *et al*. (2006). Character states are provided as ranges (minimum and maximum observed values) for each species of the ingroup. All meristic characters were transformed into ranges from 0 (= smallest observed value) to 1 (= greatest observed value). Thus the maximum weight of any meristic character is equal to that of the binary character. Missing data were assigned in the matrix by '?' if the character was considered undetermined and '-' if the character was considered not applicable.

Parsimony reconstructions were performed in TNT v1.1 (Goloboff *et al*. 2003, 2008) utilizing a combination of the parsimony ratchet (Nixon, 1999), sectorial searches, tree drifting and tree fusing (Goloboff, 1999), finding minimum length for four times with a maximum of 10,000 trees retained. Branches with a minimum length of zero were collapsed. All characters, except continuous characters, were unordered and all character transformations were equally weighted. Stability of clades was expressed as GC values (Bootstrap) and Bremer support. Bremer support was calculated from suboptimal trees with up to 20 additional steps, maxtree=10,000 trees.

Molecular analyses

DNA was extracted from tissues preserved in 96% ethanol using the Animal Tissue Direct kit (Thermo Scientific). For each sample we used PCR to amplify two mitochondrial genes: cytochrome oxidase subunit I (*COI)* and the 16S ribosomal RNA gene (*16S*); and two nuclear genes, SH3 and PX domain-containing 3-like protein (*SH3PX3*) and cardiac muscle myosin heavy chain 6 alpha (*Myh6*) (Li *et al*., 2007). PCRs were carried out in 20µl reactions containing 10-50ng DNA, 0.2µM of each primer, 0.2mM of each dNTP, 1x Buffer, 1.5µM $MgCl_2$ and 1U Platinum Taq DNA polymerase (Invitrogen, São Paulo, Brazil). PCR conditions and primers are presented in Appendix S4. PCR products were checked by electrophoresis in agarose gel, purified using ExoSap (GE Healthcare) and sequenced in both directions by ACTGene (Porto Alegre, Brazil). The forward and reverse chromatograms were assembled and visualized using the program Geneious 5.6.7 (Drummond et al. 2012b). IUPAC ambiguity codes were applied when heterozygotes or uncertainty of the nucleotide identity was detected. All sequences produced for this study have been deposited in the GenBank (XXXXX to XXXXX). DNA sequences for some ingroup and outgroup specimens were obtained from the GenBank (FJ749047.1, FJ749049.1 and FJ749052.1, Javonillo *et al*. 2010; JN712187.1, JN712188.1, JN712199.1 and JN712200.1, Casciotta *et al*., 2012; KP399682.1, KP399689.1, KP399720.1, KP399723.1, KP399734.1 - KP399736.1, KP636963.1, KP636970.1, KP637001.1, KP637004.1, KP637015.1 - KP637020.1, Hirschmann et al. 2015; HQ171412.1, HQ289120.1, Oliveira *et al*. 2011; KF209702.1, KF209704.1 - KF209705.1, KF209709.1, KF209713.1 - KF209715.1, KF209733.1 - KF209736.1, KF209739.1, KF209742.1, KF209747.1, KF209748.1, KF209753.1, KF209759.1 - KF209766.1, KF209780.1, KF209782.1 - KF209784.1, KF209797.1 - KF209806.1, KF209808.1 - KF209823.1, KF209827.1 - KF209829.1, KF209840.1 - KF209855.1, KF209906.1 - KF209911.1, KF209930.1, KF209991.1, KF209992.1, KF209995.1, KF209997.1 - KF210006.1, KF210011.1 - KF210013.1, KF210033.1, KF210034.1, KF210038.1, KF210040.1, KF210041.1, KF210058.1 - KF210061.1, KF210063.1, KF210066.1, KF210075.1, KF210085.1, KF210087.1 - KF210089.1, KF210101.1 - KF210109.1, KF210111.1 - KF210118.1, KF210122.1, KF210124.1 - KF210132.1, KF210134.1 - KF210144.1, KF210167.1 - KF210172.1, KF210241.1, KF210242.1, KF210245.1, KF210247.1 - KF210255.1, KF210260.1 - KF210262.1, KF210282.1, KF210286.1, KF210290.1 - KF210292.1, KF210305.1 - KF210308.1, KF210310.1, KF210313.1, KF210322.1, KF210328.1 - KF210332.1, KF210345.1 - KF210347.1, KF210359.1 - KF210367.1, KF210369.1 - KF210383.1, KF210386.1 -

KF210389.1, KF210402.1, KF210404.1 - KF210416.1, KF210454.1 - KF210460.1, KF210478.1, KF210535.1 - KF210536.1 and KF210540.1 - KF210548.1 Thomaz *et al*. 2015).

Phylogenetic reconstructions were performed using gene tree (using a concatenated dataset) and species tree approaches implemented in BEAST 1.8.2 (Drummond *et al*., 2012a) on the CIPRES science gateway portal (Miller *et al*., 2010). We conducted Bayesian analysis of the concatenated data set using a strict clock model approach. In the species tree approach, the two mitochondria genes *COI* and *16S* were concatenated, while the two nuclear gene alignments were allowed a different gene tree. Species tree analysis also used a strict clock model, which is a generally well-justified analysis within a species or among a few closely related species (Li & Drummond, 2012). The mtDNA data set was analyzed assuming an evolutionary rate of 0.01/site/Myr (Bermingham *et al*., 1997; Reeves & Bermingham, 2006; Ornelas-Garcia *et al*., 2008). The evolutionary rate for *Myh6* and *SH3PX3* was calibrated based on the mtDNA rate. We used the Bayesian Information Criterion (BIC) in PartitionFinder (Lanfear *et al*., 2012) and assumed a HKY+I+G substitution model for *COI, 16S* and *Myh6* and a TRN substitution model for *SH3PX3*. For the concatenated dataset, we used 100 million generations of the Markov chain Monte Carlo (MCMC), whereas for the species tree estimation, we combined two runs of 100 million generations. In all cases, samples were collected every 1,000 steps. The first 10% of the recovered topologies were discarded as burnin, and the efficiency of the chain was assessed in Tracer 1.5 (Rambaut & Drummond, 2009).

Total evidence analysis

A total evidence approach was performed by combining morphology and molecular data in the same dataset. This data set was called Stevardiinae Total Matrix, and consisted of 415 sequences for 192 terminals representing 105 Stevardiinae taxa. A total of 2,812 characters were analyzed, being 399 morphological and 2,413 molecular. A Bayesian analysis was performed using MrBayes 3.2.6 (Ronquist *et al*., 2012) on the CIPRES science gateway portal (Miller *et al*., 2010). We used different evolutionary models for each gene partition, which were selected using the BIC in PartitionFinder. For the *COI, 16S* and *Myh6* the same model (HKY+I+G) was used, while for *SH3PX3,* K80+G was the best substitution model. Morphological data was analyzed under the Mk model (Lewis, 2001), implemented in MrBayes 3.2.6, based on unordered characters and assuming a gamma-distributed rate variation across characters. We used two independent runs of 10 million MCMC generations

and four chains, sampling every 1,000 generations. The first 25% of the recovered topologies were discarded as burnin, and the efficiency of the chain was assessed using Tracer v1.5 (Rambaut & Drummond, 2009).

RESULTS

Character Descriptions

Seven characters were added herein to the list of 392 characters presented by Mirande (2010) and Mirande *et al*. (2013). Characters 396, 397 and 398 were described in Malabarba & Weitzman (2003). The other four characters 393, 394, 395 and 399 are described below. Character 395 was based on Menezes & Weitzman (2011).

393. Rhinosphenoid: (0) cartilaginous; (1) ossified.

In most *Diapoma* species the rhinosphenoid is constituted by an ossification surrounded by cartilage (state 1). However in *Diapoma alburnus* the rhinosphenoid is completely cartilaginous (state 0).

394. Posteromedial branch of supraorbital canal: (0) opens into frontal; (1) opens into parietal.

In most *Diapoma* species the posteromedial branch of supraorbital canal has an open into parietal (state 1) or have this character is coded as polymorphic.

395. Operculum shape: (0) opercle and subopercle not prolonged; (1) operculum prolonged posteriorly consisting of a triangular extension of the posteroventral field of the opercle and a posteriorly broadened posterior region of the subopercle.

The opercle prolonged was considered diagnostic for *Diapoma* for a long time, until the addition of *Diapoma terofali* to the genus (Weitzman & Fink, 1985) changing its diagnose. The prolonged operculum (state 1) is found in two species, *Diapoma speculiferum* and *Diapoma pyrrhopteryx*.

399. Anal spot: (0) absent; (1) present.

This character is an autapomorphy of *Diapoma guarani* consisting of a black spot on the anal region (state 1).

Morphological phylogenies results

The results of morphological analyses (excluding or including continuous characters) were partially congruent and supported the monophyly of the genus *Diapoma sensu* Thomaz *et al*. (2015). The analysis excluding continuous characters resulted in thirteen equally parsimonious trees (tree length 669; consistency index (CI) = 0.303; retention index (RI) = 0.734) and the consensus showed two polytomies (Appendix S5 Fig. S1). The analysis with continuous characters resulted in one tree with a higher consistency index (tree length 560.346; CI = 0.361 (RI) = 0.780), and a completely resolved phylogeny for all species of *Diapoma* (Fig. 1).

In both hypotheses, *D. alburnus* appears as sister group to other species of the genus, followed successively by a clade containing *D. dicropotamicus* and *D. itaimbe* as a sister group to the remaining species. Both analyses suggested that the other species of the genus grouped in four clades: *Diapoma* sp. Iguaçu; *Diapoma* sp. + *D. guarani*; (*D. speculiferum* + *D. pyrrhopteryx*) + (*Diapoma terofali* + *D. thauma*); and a fourth clade containing the remaining five species. These four clades form an unresolved phylogeny in the analysis using discrete characters, but their relationships are fully resolved in the analysis with continuous characters, though with a low support for the clade containing (*Diapoma* sp. + *D. guarani*) ((*D. speculiferum* + *D. pyrrhopteryx*) + (*Diapoma terofali* + *D. thauma*)). The fourth clade with five species also shows a polytomy in the analysis using discrete characters, but is fully solved when continuous characters are added to the analysis ((*D. obi* + *D. uruguayensis*) (*D. lepiclastus* (*D. tipiaia* + *D. alegretensis*))).

Molecular phylogenies results

A total of 111 sequences from two mitochondrial (*COI* and *16S*) and two nuclear loci (*Myh6* and *SH3PX3*) were generated from 48 individuals. Some markers could not be successfully amplified and sequenced for all specimens due to technical issues or low DNA quality and could not be obtained from the GenBank. A total of 220 sequences from 66 taxa, being 59 of ingroup and seven of outgroup, composes the matrix. The concatenated alignment contains 2,401 sites, of which 284 are variable. Mitochondrial and nuclear DNA sequences could be obtained for 98 % and 76 % of the ingroup taxa, respectively.

The results of both molecular analyses (species tree and concatenated) were congruent (Fig. 2; Appendix S5 Fig. S2) and supported the monophyly of the *Diapoma sensu* Thomaz *et al*. (2015). The concatenated analysis showed four major clades with high support values (1).

The first clade has *D. alburnus* as sister to a *D. itaimbe* plus *D. dicropotamicus* clade. The second clade is composed by *D. tipiaia*, *Diapoma* sp. Iguaçu, *Diapoma* sp. and a smaller clade containing *D. guarani* and *D. obi* where *D. guarani* is not a monophyletic species. The third clade is composed by *D. speculiferum*, *D. pyrrhopteryx* and *D. terofali* and the fourth by *D. alegretensis*, *D. lepiclastus* and *D. uruguayensis*, which does not seem to be monophyletic species. *Diapoma thauma* appears as the sister group of the fourth clade, but with a relatively low support (0.8). The species tree showed the same four major clades that the gene tree, but with *D. itaimbe* as sister to the *D. alburnus* and *D. dicropotamicus* clade (Fig. 2). Again, the lowest support was observed in the position of *D. thauma* as sister group to *D. alegretensis*, *D. lepiclastus* and *D. uruguayensis* clade.

Total evidence analysis results

The total evidence approach (Fig. 3) also supported the monophyly of the *Diapoma sensu* Thomaz *et al*. (2015). Likewise the concatenated and species tree approaches, *Diapoma alburnus*, *D. dicropotamicus* and *D. itaimbe* form a clade that is sister to the remaining species. The three remaining clades are similar to those found in the concatenated and species tree approaches, but the relationship of *D. thauma* was similar to that observed in the morphological phylogenies.

List of synapomorphies

The synapomorphies of the most inclusive *Diapoma* clade are listed below according to the total evidence approach. Convergences and reversions are listed only within *Diapoma*. In the following section, clades are discussed following the nodes in the cladogram depicted in Fig. 3. The number of the characters supporting the monophyly of each node is given in parentheses after its definition, according to the list of characters provided in Appendix S2.

Node 264: *Diapoma.*

The genus *Diapoma* includes the species proposed by Thomaz *et al*. (2015) with the addition of *D. thauma* and *Diapoma* sp.. This clade is recovered here in all phylogenetic approaches based on molecular, morphological, and total evidence datasets.

Synapomorphies

1. Position of the opening on neurocranium communicating with laterosensory canal of sixth infraorbital: (77: 1 > 0) between frontal and pterotic.

2. Posterior extent of ventral process of quadrate: (151: 0 > 1) falling short of posterior margin of symplectic.

Reversed in *D. obi*.

3. Development of transverse process of neural arch of third vertebra: (219: 0 > 1) well developed and extending beyond anterior margin of tripus.

Reversed in *D. obi* and *D. terofali.*

4. Process of scapula forming anterior border of scapular foramen: (244: 0 > 1): absent.

5. Number of branched pelvic-fin rays: (258: 1 > 0) six or less.

6. Longitudinal position of insertion of adductor mandibulae tendon on dentary: (330: 1 > 0) on vertical through posterior half of Meckelian cartilage.

Node 263: *Diapoma alburnus, Diapoma dicropotamicus* and *Diapoma itaimbe.*

The monophyly of the clade including the species *D. alburnus*, *D. dicropotamicus* and *D. itaimbe* was previously proposed by Thomaz *et al*. (2015) was supported only by molecular evidence. Thus, no morphological synapomorphies were found for this clade.

Autapomorphies of *Diapoma alburnus.*

1. Bony hooks on base of pelvic-fin rays of adult males: (313: 1 > 0) absent, or in small number compared to those in the segmented portion of rays.

Paralleled in *C. obi* and in the clade (*D. pyrrhopteryx* + *D. speculiferum*).

2. Bony hooks on last pelvic-fin ray of adult males: (314: 1 > 0) absent or reduced in number.

Paralleled in node 297 and reversed in *C. obi*

3. Rhinosphenoid: (393: 1 > 0) cartilaginous.

Node 271: *Diapoma dicropotamicus* and *Diapoma itaimbe.*

This clade was originally proposed by Malabarba & Weitzman (2003) and posteriorly corroborated by Thomaz *et al*. (2015). Herein it was recovered in all phylogenetic approaches except in the species tree approach. The “synapomorphy 2” that was proposed by Malabarba & Weitzman (2003) to support their relationship was corroborated here.

Synapomorphies

1. Form of fourth infraorbital: (67: 0 > 1) longer dorsoventrally than longitudinally.

2. Black pigmented adipose fin: (397: 0 > 1) present.

Autapomorphies of *Diapoma dicropotamicus.*

1. Bony lamellae between second and third basibranchials: (184: 1 > 0) absent.

Paralleled in *D. alegretensis* and *D. tipiaia*.

Autapomorphies of *Diapoma itaimbe.*

No morphological autapomorphies were found for this species.

Node 282: *Diapoma* except *D. alburnus*, *D. itaimbe* and *D. dicropotamicus.*

The largest clade previously proposed by Thomaz *et al*. (2015) is also recovered in this study in all phylogenetic approaches.

Synapomorphies

1. Lateral line: (91: 0 > 1) (1) interrupted.
2. Canal of lateral line on caudal-fin membrane: (92: 1 > 0) absent.
3. First postcleithrum: (247: 0 > 1) absent.

Reversed in *Diapoma* sp. Iguaçu.

Node 289: *Diapoma tipiaia, Diapoma sp.* Iguaçu*, Diapoma* sp*., D. guarani* and *D. obi*

The monophyly of a clade equivalent to this grouping was not proposed in previous phylogenies. This clade is recovered only with molecular and total evidence approaches. Thus there is no morphological synapomorphies for this clade.

Autapomorphies of *Diapoma tipiaia*

1. Contact between frontals anteriorly to frontal fontanel: (21: 0 > 1) present.
2. Form of quadrate (150: 0 > 1) with anterodorsal portion equal or longer than ventral region.

Paralleled in *D. thauma*.

3. Bony lamellae between second and third basibranchials: (184: 1 > 0) absent.

Paralleled in *D. alegretensis* and *D. dicropotamicus*.

4. Dorsal development of third postcleithrum: (251: 0 > 1) not projecting dorsally to posterior region of scapula.

Paralleled of synapomorphy of node 300 and reversal in *D. speculiferum*.

5. Posteromedial branch of supraorbital canal: (394: 1 > 0) opens into frontal.

Paralleled in *D. thauma*.

Node 292: *Diapoma* sp. Iguaçu, *Diapoma* sp., *D. guarani* and *D. obi*

This monophyletic group was not proposed in previous phylogenies. This clade was recovered with low support and only with molecular and total evidence approaches. Thus, there are no morphological synapomorphies for this clade.

Autapomorphies of *Diapoma sp.* Iguaçu

1. First postcleithrum: (247: 1 > 0) present.
2. Position of ventral margin of posttemporal: (252: 1 > 0) anterior to lateral margin of epioccipital.

Paralleled as a synapomorphy of node 279 and in *D. speculiferum*.

3. Anal-fin position: (284: 1 > 0) posterior or almost posterior to vertical through last dorsal-fin ray.

Paralleled in *C. obi*.

Node 297: *Diapoma* sp., *D. guarani* and *D. obi*

This minor clade was recovered only with molecular and total evidence approaches, but with high support in both of them.

Synapomorphies

1. Abrupt decrease in the size of dentary teeth: (148: 0 > 1) present.
2. Contact between lamella on anterior portion of first basibranchial with lamella on posterior portion of second basibranchial: (183: 0 > 1) present.

Reversed in *D. obi*.

3. Bony hooks on last pelvic-fin ray of adult males: (314: 1 > 0) absent or reduced in number.

Reversed in *D. obi.*

4. Radii on scales: (320: 1 > 0) absent or reduced in number.

Reversed in *D. obi*.

5. Sclerotic bones: (350: 0 > 1) two bones separated by cartilages.

Autapomorphies of *Diapoma* sp.

1. Sperm nuclei: (359: 1 > 0) spherical.

Node 296: *D. guarani* and *D. obi*

This minor clade was previously proposed by Thomaz *et al*. (2015) and was recovered

in this study by only with molecular and total evidence approaches. However, *D. guarani* does not appear as monophyletic on concatenated and total evidence approaches.

Synapomorphies

1. Gill-derived gland on males: (352: 1 > 0) absent.

Paralleled in *D. terofali*.

2. Testicle with three partitions: (360: 0 > 1) (1) present.

Autapomorphies of *D. obi*

1. Ventral extent of third infraorbital: (64: 1 > 0) not reaching horizontal arm of preopercle, at least anteriorly.

Paralleled of synapomorphy 1 of node 301.

2. Separation between posterior dentary teeth: (147: 1 > 0) less than width of these teeth.

3. Posterior extent of ventral process of quadrate: (151: 1 > 0) reaching vertical through posterior margin of symplectic.

Reversal of synapomorphy 2 of node 264.

4. Contact between lamella on anterior portion of first basibranchial with lamella on posterior portion of second basibranchial: (183: 1 > 0) absent.

Reversal of synapomorphy 2 of node 297.

5. Cartilages anterior to basihyal: (188: 0 > 1) two well developed blocks of cartilage.

6. Development of transverse process of neural arch of third vertebra: (219: 1 > 0) not reaching anterior margin of tripus.

Reversal of synapomorphy 3 of node 264.

7. Anal-fin position: (284: 1 > 0) posterior or almost posterior to vertical through last dorsal-fin ray.

Paralleled in *Diapoma* sp. Iguaçu.

8. Bony hooks on base of pelvic-fin rays of adult males: (313: 0 > 1) as numerous as on segmented portion of rays.

Paralleled in *D. alburnus* and reversal of synapomorphy 2 of node 301.

9. Bony hooks on last pelvic-fin ray of adult males: (314: 0 > 1) as numerous as in other rays.

Paralleled in *D. alburnus* and reversal of synapomorphy 3 of node 297.

10. Radii on scales: (320: 0 > 1) present and numerous on most scales.

Reversal of synapomorphy 4 of node 297.

11. Number of cusps of anterior dentary teeth: (380: 0 > 1) five or more.

Reversed in *D. thauma*.

12. Cartilage-filled region anterior to scapular foramen: (387: 0 > 1) reduced by expansions of coracoid and cleithrum.

13. Intense blue color in mature specimens: (396: 0 > 1) present.

Node 281: *Diapoma thauma, D. terofali, D. speculiferum, D. pyrrhopteryx, D. uruguayensis, D. alegretensis* and *D. lepiclastus.*

This is a novel clade, which has not been proposed in previous phylogenies. This clade is recovered only with molecular and total evidence approaches but in both of them with high support. There are, however, morphological synapomorphies for this clade.

Synapomorphies

1. Number of branched anal-fin rays: (288: 0 > 1) 25 or more.

Node 300: *Diapoma thauma, D. terofali, D. speculiferum, D. pyrrhopteryx*

This clade, previously proposed by Thomaz *et al*. (2015), was recovered here in total evidence and morphological approaches only.

Synapomorphies

1. Rows of gill rakers on second ceratobranchial: (193: 1 > 0) one.

Paralleled in *D. alegretensis*.

2. Dorsal development of third postcleithrum: (251: 0 > 1) not projected dorsally to posterior region of scapula.

Paralleled in *D. tipiaia* and reversed in *D. speculiferum*.

3. Glandular tissue of granular appearance on caudal fin of mature males: (353: 0 > 1) present.

4. Hypertrophied ventral caudal-peduncle squamation: (354: 0 > 1) (1) present.

5. Caudal gland cells consisting of modified mucous cells: (355: 0 > 1) (1) present.

Autapomorphies of *Diapoma thauma*

1. Lateral coverage of dilator fossa by sixth infraorbital: (69: 1 > 0) almost complete, at least in its ventral border.

Paralleled in *D. pyrrhopteryx*.

2. Form of quadrate: (150: 0 > 1) with anterodorsal portion equal or longer than ventral region.

Paralleled in *D. tipiaia*.

3. Palatine foramen: (173: 0 > 1) present and very conspicuous.

4. Coracoid foramen: (243: 0 > 1) well developed.

5. Number of ventral procurrent caudal-fin rays: (302: 0 > 1) 12 or more.

Paralleled in *D. uruguayensis*.

6. Ventral procurrent caudal-fin rays of adult males: (303: 0 > 1) projecting ventrally through peduncle musculature and skin.

7. Bony hooks on first pelvic-fin ray of adult males: (315: 0 > 1) present.

Paralleled in *D. lepiclastus*.

8. Number of cusps of anterior dentary teeth: (380: 1 > 0) three or fewer.

Reversed in *D. obi*.

9. Posteromedial branch of supraorbital canal: (394: 1 > 0) opens into frontal.

Paralleled in *D. tipiaia*.

Node 302: *D. terofali, D. speculiferum, D. pyrrhopteryx*

This clade was previously proposed by Thomaz *et al*. (2015) and was recovered in this study by total evidence and molecular phylogenetic approaches, except by morphological phylogenies. There were no morphological synapomorphies for this clade.

Autapomorphies of *D. terofali.*

1. Development of transverse process of neural arch of third vertebra: (219: 1 > 0) not reaching anterior margin of tripus.

Reversal of synapomorphy 3 of node 264 and paralleled in *C. obi*.

2. Gill-derived gland on males: (352: 1 > 0) absent.

Paralleled in node 296.

Node 301: *D. speculiferum* and *D. pyrrhopteryx.*

This relationship was previously proposed by Thomaz *et al*. (2015) and was recovered here with low support in total evidence and morphological phylogenies. The molecular phylogenies showed *D. pyrrhopteryx* more related closely with *D. terofali*, with high support.

Synapomorphies

1. Ventral extent of third infraorbital: (64: 1 > 0) not reaching horizontal arm of preopercle, at least anteriorly.

Paralleled in *C. obi*.

2. Bony hooks on base of pelvic-fin rays of adult males: (313: 1 > 0) absent, or in small number compared to on segmented portion of rays. Reversed in *D. alburnus* and *D. obi*.

3. Operculum shape: (395: 0 > 1) operculum prolonged posteriorly consisting of a triangular extension of the posteroventral field of the opercle and a posteriorly broadened posterior region of the subopercle.

Autapomorphies of *D. pyrrhopteryx.*

1. Lateral coverage of dilator fossa by sixth infraorbital: (69: 1 > 0) almost complete, at least in its ventral border. Paralleled in *D. thauma*.

2. Relative length of palatine: (172: 0 > 1) distinctly longer than one-half length of ectopterygoid.

3. Parietal branch of supraorbital laterosensory canal: (386: 1 > 0) extended posteriorly to middle region of parietal.

Autapomorphies of *D. speculiferum.*

1. Dorsal development of third postcleithrum: (251: 1 > 0) projects dorsally to posterior region of scapula.

Reversal of synapomorphy 2 of node 300 and reversed in *D. tipiaia*.

2. Position of ventral margin of posttemporal: (252: 1 > 0) anterior to lateral margin of epioccipital. Paralleled of synapomorphy of node 279 and in *Diapoma* sp.Iguaçu.

Node 280: *D. uruguayensis, D. alegretensis and D. lepiclastus.*

A similar clade to this grouping was proposed by Malabarba & Weitzman (2003). Herein this relationship was recovered by total evidence and molecular phylogenies. *Diapoma uruguayensis* does not appear as monophyletic on the concatenated tree, but in the total evidence approach the monophyly of this species was recovered.

Synapomorphies

1. Shape of anal fin male: (398: 0 > 2) deeply convex margin.

Reversed in *D. lepiclastus*.

Autapomorphies of *D. uruguayensis*

1. Bony lamella dorsal to fourth basibranchial: (185: 0 > 1) absent.
2. Coracoid foramen: (243: 0 > 1) well developed.

Paralleled in *D. thauma*.

Node 279: *D. alegretensis* and *D. lepiclastus*

This is a novel clade, which has not been proposed in previous phylogenies. In this study, this clade was recovered only in the total evidence phylogeny. Both molecular phylogenies showed *D. lepiclastus* more related closely with *D. uruguayensis*, with *D. alegretensis* as sister to them. On the morphological phylogenies *D. alegretensis* was more related to *D. tipiaia*, with *D. lepiclastus* as sister to them.

Synapomorphies

1. Position of ventral margin of posttemporal: (252: 1 > 0) anterior to lateral margin of epioccipital.

Paralleled in *D*. sp. Iguaçu and *D. speculiferum*.

Autapomorphies of *D. alegretensis*

1. Bony lamellae between second and third basibranchials: (184: 1 > 0) absent.

Paralleled in *D. tipiaia* and *D. dicropotamicus*.

2. Rows of gill rakers on second ceratobranchial: (193: 1 > 0) one.

Paralleled of synapomorphy of 1 of node 300.

Autapomorphies of *D. lepiclastus*

1. Bony hooks on first pelvic-fin ray of adult males: (315: 0 > 1) (1) present.

Paralleled in *D. thauma*.

2. Shape of anal fin male: (398: 2 > 0) anal fin with a concave distal bordes.

Reversal of a synapomorphy in node 280.

DISCUSSION

The history of the classification of the species currently harbored in *Diapoma* is interesting and, in some ways, reflects what may be expected to happen in characid

classification in the future. In particular, the taxonomic history of its earliest described species, *Tetragonopterus alburnus* Hensel, 1870 is illustrative of the new trends in characid taxonomy. *Diapoma alburnus* was described twice, *Astyanax hasemani* Eigenmann, 1914 being a junior synonym. Both nominal species, however, were considered as valid in the American Characidae monography of Eigenmann (1927), and were assigned to different genera, as *Bryconamericus alburnus* and *Astyanax hasemani*. This odd situation was detected later, and justified on the grounds that this species presents a polymorphic condition of the character then in use to diagnose *Astyanax* and *Bryconamericus*, which was related to the presence of five or four teeth in the inner series of the premaxilla, respectively (Malabarba, 1983, 1987). Similar problems with the traditional classification of Eigenmann to distinguish characid genera were discussed by Fink (1979: "These problems arise primarily because the system now in use, which dates from the work of Eigenmann (1917), is obsolescent and no longer able to incorporate the diversity of the fishes it was intended to classify") and Weitzman & Fink (1983), who merged species from three genera and two subfamilies in a single genus after a detailed comparative osteological study and a phylogenetic analysis of these taxa. However, the lack of a general framework stablishing phylogenetic relationships among characid taxa precluded the proposal of new classifications for characid genera and species for a long time (Weitzman & Malabarba, 1998). In the case of *Diapoma alburnus*, even though *Astyanax hasemani* (=*Diapoma alburnus*) was considered possibly related to the glandulocaudines *Diapoma speculiferum* and *Glandulocauda terofali* (=*Diapoma terofali*), these species were left in separate genera until a phylogenetic analysis of characid became available (Malabarba, 1983).

In the next two decades through the end of the Twenty Century, a few phylogenies were published on some groups of characid fishes. These clades were summarized in the supertree presented by Malabarba & Weitzman (2003), who left unclassified a large number of genera considered as *incertae sedis* in Characidae due to the lack of information abut their phylogenetic relationships. Similarly, nearly two thirds of Characidae species were listed as *incertae sedis* by Lima *et al*. (2003), away from well supported subfamilies such as Agoniatinae (Lima & Zanata, 2003), Clupeacharacinae, Bryconinae (Lima, 2003), Iguanodectinae (Moreira, 2003), Serrasalminae (Jégu, 2003), Aphyocharacinae (Lima, 2003), Characinae (Lucena & Menezes, 2003), Stethaprioninae, Tetragonopterinae (Reis, 2003), Rhoadsiinae (Cardoso, 2003), Cheirodontinae (Malabarba, 2003) and Glandulocaudinae (Weitzman, 2003). The supertree of Malabarba & Weitzman (2003) brought two other novelties: the proposition of a large subclade containing all the species lacking a supraorbital

bone (latter proposed as Characidae *stricto sensu*; Oliveira *et al.*, 2011), and a smaller subclade of genera with four teeth in the inner series of the premaxilla and ii + 8 dorsal-fin rays (latter proposed as Stevardiinae). Both *Diapoma* and the new genus *Cyanocharax*, containing *C. alburnus* in a new combination, were proposed to belong to this subclade (named Clade A therein). *Diapoma*, however, was by that time included in the tribe Diapomini, subfamily Glandulocaudinae, which are groups strongly supported by primary homologies related to primary and secondary sexual characters (the caudal-fin gland and insemination) (e.g., Weitzman & Menezes, 1998; Menezes & Weitzman, 2011). Although the monophyly of Glandulocaudinae and its tribes were long supported by direct polarity decisions, their monophylies remained to be tested in global analyses including other characid taxa and characters not related to the sexual system.

By the end of the first decade of this century, a few and important papers have been published bringing taxa-inclusive analyses to test existing taxonomic groups based on Eigenmann´s classification as well as groups proposed in the recent years based on phylogenetic arguments (Javonillo *et al*., 2010, Mirande, 2010, Oliveira *et al*., 2011). All these papers pointed out the non-monophyly of the glandulocaudines (*sensu* Weitzman & Menezes, 1998) and showed that its genera may be more related to some genera lacking insemination and the caudal fin gland. One example was *Diapoma*, whose species were recovered as closely related to *Cyanocharax alburnus* and the other species of the genus in molecular analyses (Javonillo *et al*., 2010). Such result was coorroborated by Thomaz *et al*. (2015) based on molecular data, but not by studies using morphological data (Mirande, 2010; Mirande *et al*., 2013).

An integrative approach combining morphological and molecular information seems to be necessary in this case to establish the phylogenetic relationships among the species of *Diapoma* and of those assigned to its junior synonym, *Cyanocharax*. In agreement with Javonillo *et al*. (2010) and Thomaz *et al*. (2015), the monophyly of *Diapoma* (including *Cyanocharax)* was recovered in this study in separate as well as total evidence analysis of molecular and morphological data. The number of branched pelvic-fin rays (i+6) proposed by Thomaz *et al*. (2015) as a synapomorphy for *Diapoma* clade was tested here and corroborated. Moreover five additional synapomorphies are listed for the *Diapoma* clade, but these synapomorphies are not exclusive for this group.

The relationships among the species of *Diapoma* recovered by morphological and molecular analyses were incongruent, that is, no internal relationship was repeated. However, the molecular phylogenies had higher support for the clades than the morphological

phylogenies. The limited morphological variation among species together with evolutionary convergence observed in this study resulted in extensive homoplasy and decreased phylogenetic resolution. According to Mirande (2010) these morphological characters are highly homoplastic and although useful at some level are not sufficient to diagnose generic or suprageneric clades. Adding other characters to the matrix is still not sufficient to diagnose some clades based on morphological data. Therefore more morphological investigation is required to establish morphological characters useful for diagnosing the internal clades in this group.

In general, total evidence phylogeny showed higher support for internal relationships of *Diapoma* species and it was possible to trace morphological synapomorphies. However, the disparity in terms of numbers of traits and phylogenetic signal comparing morphological data sets (typically containing fewer traits) and molecular datasets is an issue that must be taken care, as even a large morphological data set may be overwhelmed by a modest molecular data set (Lee *et al*., 2013; O'Leary *et al*., 2013). The total evidence analysis based on Bayesian inference seem to perform better then Maximum Likelihood regardless of the amount of missing data (Guilherme & Cooper, 2016), which has been observed also in empirical data (e.g. Arcila *et al*., 2015). However the final result of the total evidence phylogeny showed a strong influence of the molecular data, such that the total evidence phylogeny is much closer to the molecular phylogeny, as stated by Lee *et al*. (2013) and O'Leary *et al*. (2013). Nevertheless, we can detect a few instances where the morphological data gave decisive support for some relatioships, such as the clade including *Diapoma thauma* + *D. terofali* + *D. speculiferum* + *D. pyrrhopteryx* and the clade composed by *D. speculiferum* + *D. pyrrhopteryx*.

Internal relationships in *Diapoma* were partially congruent with prior phylogenic hypothesis (Thomaz *et al*. 2015). Three internal clades were similar with this previous proposal: (*D. alburnus* + *D. itaimbe* + *D. dicropotamicus*), (*D. guarani* + *D. obi*), and (*D. terofali* + *D. pyrrhopteryx* + *D. speculiferum*). In our study, *D. pyrrhopteryx* is sister to *D. speculiferum* based on homologous opercular modifications, as proposed by Menezes & Weitzmann (2011). In addition, *D. itaimbe* and *D. dicropotamicus* are sister based on two synapomorphies, one of them being the black pigmented adipose fin as proposed by Malabarba & Weitzman (2003). New findings from our study were *D. alegretensis* sister to *D. lepiclastus* with *D. uruguayensis* sister to this clade, and the phylogenetic position of *D. tipiaia* and *Diapoma* sp. Iguacu. However, the phylogenetic hypothesis presented in this study still has low support values in a few clades, such that more studies are needed to address these

clades. To conclude, our study presented a new hypothesis of internal relationships among *Diapoma* species, and is the first to do so based on morphological and molecular data simultaneously.

Comparative material

Diapoma alburnus (Hensel, 1870): UFRGS 999, 1 (c&s), 30.01 mm SL, Brazil, Rio Grande do Sul, Guaíba, Arroio dos Ratos; UFRGS 4598, 2 (c&s), 34.52 – 35.33 mm SL, Brazil, Rio Grande do Sul, Tramandaí drainage, Lagoa Emboaba; *Diapoma itaimbe* (Malabarba & Weitzman, 2003): UFRGS 15381, 1 (c&s), 52.13 mm SL, Brazil, Santa Catarina, Treviso, Araranguá drainage, Rio Mãe Luzia; UFRGS 12561, 2 (c&s), 45.85 – 49.96 mm SL, Brazil, Santa Catarina, Treviso, Araranguá drainage, Rio Manin; MCP 14788, 3 (c&s), 40.40 – 44.63 mm SL, Brazil, Santa Catarina, Praia Grande, Mampituba drainage, Arroio Facão; *Diapoma dicropotamicus* (Malabarba & Weitzman, 2003): UFRGS 13949, 2 (c&s), 45.15 – 46.20 mm SL, Brazil, Rio Grande do Sul, Marques de Souza, Arroio Tamanduá, MCP 19510, 2 (c&s), 37.30 – 40.16 mm SL, Brazil, Rio Grande do Sul, Cruzeiro do Sul, Rio Taquari; *Diapoma alegretensis* (Malabarba & Weitzman, 2003): UFRGS 10417, 3 (c&s), 35.29 – 42.73 mm SL, Brazil, Rio Grande do Sul, Entre Ijuís, Rio Ijuizinho, MCP 11232, paratypes, 3 (c&s), 40.41 – 43.68 mm SL, Brazil, Rio Grande do Sul, Rosário do Sul, tributary of Rio Ibirapuitã; *Diapoma uruguayensis* (Messner, 1962): UFRGS 7793, 2 (c&s), 42.18 – 49.62 mm SL, Uruguay, Paysandu, Arroio Carpinchuri, MCP 16382, paratypes, 2 (c&s), 42.15 – 45.28 mm SL, Brazil, Rio Grande do Sul, Santana do Livramento, Rio Sarandi; *Diapoma lepiclastus* (Malabarba & Weitzman, 2003): UFRGS 10917, 2 (c&s), 38.26 – 41.39 mm SL, Brazil, Santa Catarina, Itapiranga, Rio Dourados, MCP 14557, paratypes, 1 (c&s), 42.96 mm SL, Brazil, Rio Grande do Sul, Palmitinho, Arroio Lageado União; *Diapoma tipiaia* (Malabarba & Weitzman, 2003): MCP 22712, paratypes, 1 (c&s), 38.49 mm SL, Brazil, Rio Grande do Sul, Júlio de Castilhos, Arroio Felício; *Diapoma* sp. Iguaçu: MCP 41543, 2 (c&s), 53.08 – 58.95 mm SL, Brazil, Paraná, Reservatório Salto Osório; *Diapoma pyrrhopteryx* Menezes & Weitzman, 2011: UFRGS 13050, 1 (c&s), 58.92 mm SL, Brazil, Rio Grande do Sul, Boa Vista do Cadeado, Rio Ijuizinho; *Diapoma terofali* (Géry, 1964): UFRGS 2074, 1 (c&s), 40.11 mm SL, Brazil, Rio Grande do Sul, Bagé, Rio Negro, UFRGS 2076, 1 (c&s), 41.23 mm SL, Brazil, Rio Grande do Sul, Bagé, Rio Negro; *Diapoma speculiferum* Cope, 1894: UFRGS 2063, 1 (c&s), 37.87 mm SL, Brazil, Rio Grande do Sul, Guaíba, Arroio dos Ratos, UFRGS 2067, 1 (c&s), 30.84 mm SL, Brazil, Rio Grande do Sul, Santo Antônio da Patrulha; *Diapoma thauma* Menezes & Weitzman, 2011: UFRGS 11848, 2 (c&s), 39.73 – 40.54 mm SL, Brazil,

Rio Grande do Sul, Guaporé, Rio Carreiro, UFRGS 8948, 2 (c&s), 37.34 – 40.98 mm SL, Brazil, Rio Grande do Sul, Cotiporã, Rio Carreiro; *Diapoma guarani* (Mahnert & Géry, 1987): UFRGS 8480, 5 (c&s), 33.86 – 34.93 mm SL, Brazil, Rio Grande do Sul, Uruguaiana, Barragem Sanchuri; *Diapoma* sp.: UFRGS 8464, 2 (c&s), 33.02 – 34.65 mm SL, Brazil, Rio Grande do Sul, Bagé, Sanga Cinco Salsos, UFRGS 8122, 2 (c&s), 32.43 – 32.81 mm SL, Uruguay, Rivera, Arroio Corrales.

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We are grateful to Carlos A. S. Lucena (PUCRS) for material loan, Casey B. Dillman for assistance with the MrBayes software and Juliana M. Wingert, Juliano Ferrer and Fernando R. Carvalho for help with morphological characteres. Thanks for financial support from CNPq (Proc. 160848/2011-3 to AH and 300705/2010-7; 477318/2012-6 to LRM).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Complete list of the analyzed discrete morphological characters.

Appendix S2 Complete discrete morphological data matrix.

Appendix S3 Complete list and data matrix of continuous morphological characters.

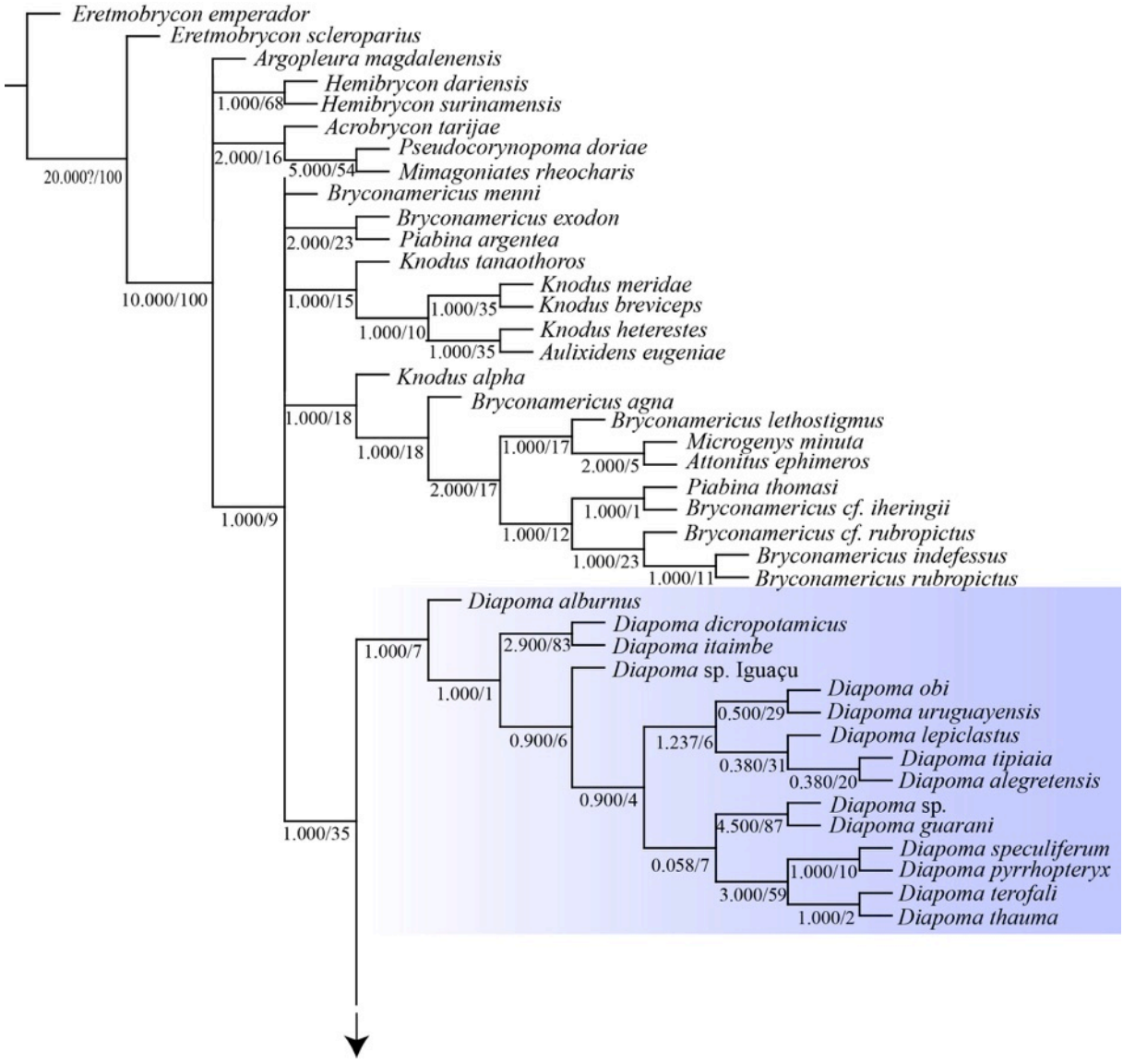
Appendix S4 Additional table (S1).

Appendix S5 Additional figures (S1 and S2).

Fig. 1. Parsimony-based cladogram including continuous and discrete morphological characters, showing the phylogenetic relationships in *Diapoma* and the remaining Stevardiinae. Bremer/bootstrap support values are shown in the nodes.

Fig. 2. Species tree based on molecular data showing the phylogenetic relationships in *Diapoma*. Posterior probabilities values are shown in the nodes.

Fig. 3. Bayesian cladogram based in a total evidence approach. Node numbers correspond to those discussed in the text, and the stars correspond to the posterior probabilities for each node, as shown in the legend.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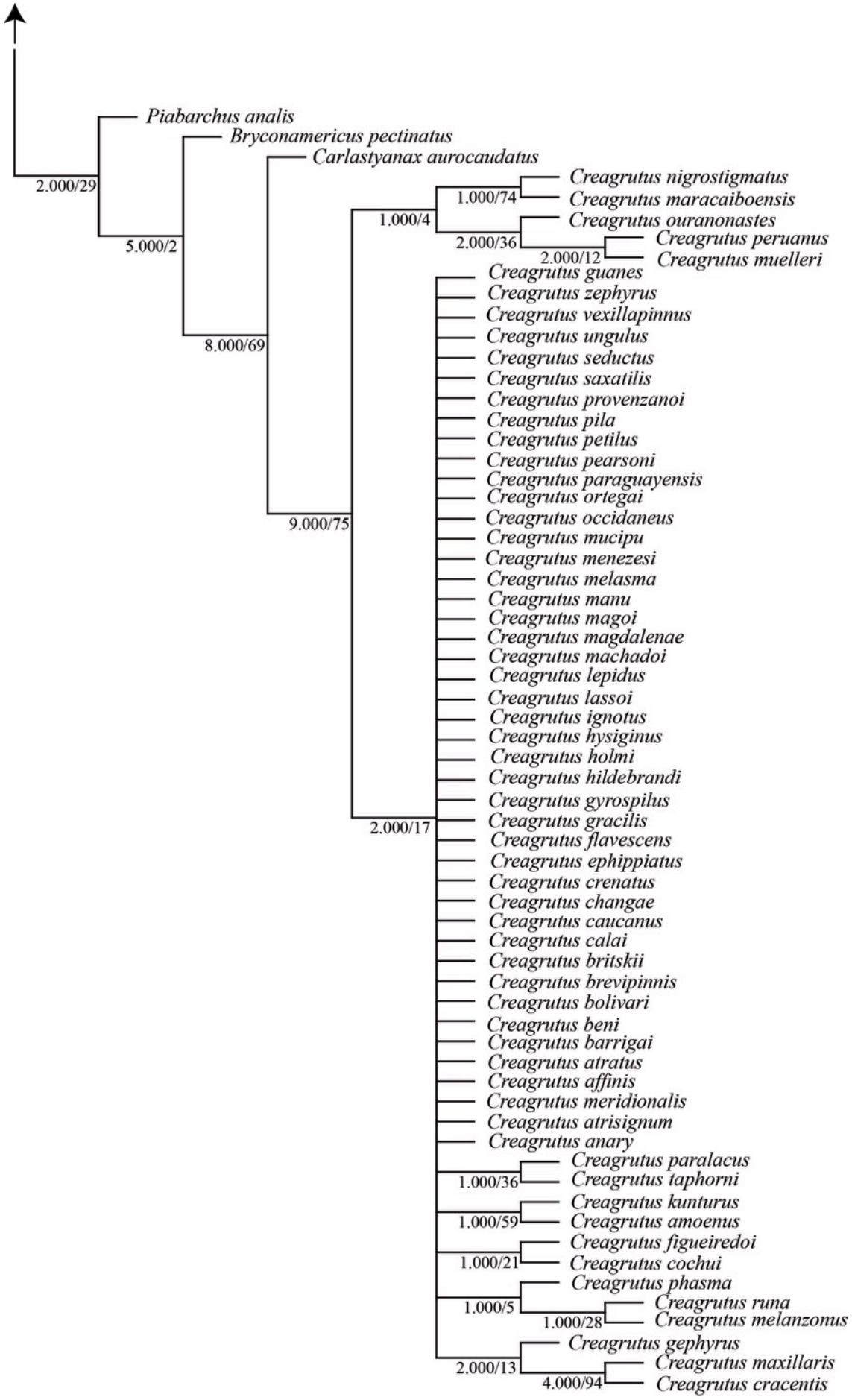
Hemibrycon dariensis
1.000/68
Hemibrycon surinamensis
Acrobrycon tarijae
2.000/16
Pseudocorynopoma doriae
5.000/54
Mimagoniates rheocharis
20.000?/100
Bryconamericus menni
Bryconamericus exodon
2.000/23
Piabina argentea
Knodus tanaothoros
10.000/100
1.000/15
Knodus meridae
1.000/35
Knodus breviceps
1.000/10
Knodus heterestes
1.000/35
Aulixidens eugeniae
Knodus alpha
1.000/18
Bryconamericus agna
1.000/18
Bryconamericus lethostigmus
1.000/17
Microgenys minuta
2.000/5
Attonitus ephimeros
2.000/17
Piabina thomasi
1.000/1
Bryconamericus cf. iheringii
1.000/9
1.000/12
Bryconamericus cf. rubropictus
1.000/23
Bryconamericus indefessus
1.000/11
Bryconamericus rubropictus
Diapoma alburnus
Diapoma dicropotamicus
1.000/7
2.900/83
Diapoma itaimbe
Diapoma sp. Iguaçu
1.000/1
Diapoma obi
0.500/29
Diapoma uruguayensis
0.900/6
1.237/6
Diapoma lepiclastus
0.380/31
Diapoma tipiaia
0.380/20
Diapoma alegretensis
0.900/4
Diapoma sp.
4.500/87
Diapoma guarani
1.000/35
0.058/7
Diapoma speculiferum
1.000/10
Diapoma pyrrhopteryx
3.000/59
Diapoma terofali
1.000/2
Diapoma thauma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Creagrutus peruanus
Creagrutus muelleri
Creagrutus guanes
Creagrutus zephyrus
Creagrutus vexillapinnus
Creagrutus ungulus
Creagrutus seductus
Creagrutus saxatilis
Creagrutus provenzanoi
Creagrutus pila
Creagrutus petilus
Creagrutus pearsoni
Creagrutus paraguayensis
Creagrutus ortegai
Creagrutus occidaneus
Creagrutus mucipu
Creagrutus menezesi
Creagrutus melasma
Creagrutus manu
Creagrutus magoi
Creagrutus magdalenae
Creagrutus machadoi
Creagrutus lepidus
Creagrutus lassoi
Creagrutus ignotus
Creagrutus hysiginus
Creagrutus holmi
Creagrutus hildebrandi
Creagrutus gyrospilus
Creagrutus gracilis
Creagrutus flavescens
Creagrutus ephippiatus
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Creagrutus caucanus
Creagrutus calai
Creagrutus britskii
Creagrutus brevipinnis
Creagrutus bolivari
Creagrutus beni
Creagrutus barrigai
Creagrutus atratus
Creagrutus affinis
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Creagrutus atrisignum
Creagrutus anary
Creagrutus paralacus
Creagrutus taphorni
Creagrutus kunturus
Creagrutus amoenus
Creagrutus figueiredoi
Creagrutus cochui
Creagrutus phasma
Creagrutus runa
Creagrutus melanzonus
Creagrutus gephyrus
Creagrutus maxillaris
Creagrutus cracentis
2.000/29
5.000/2
8.000/69
9.000/75
1.000/4
1.000/74
2.000/36
2.000/12
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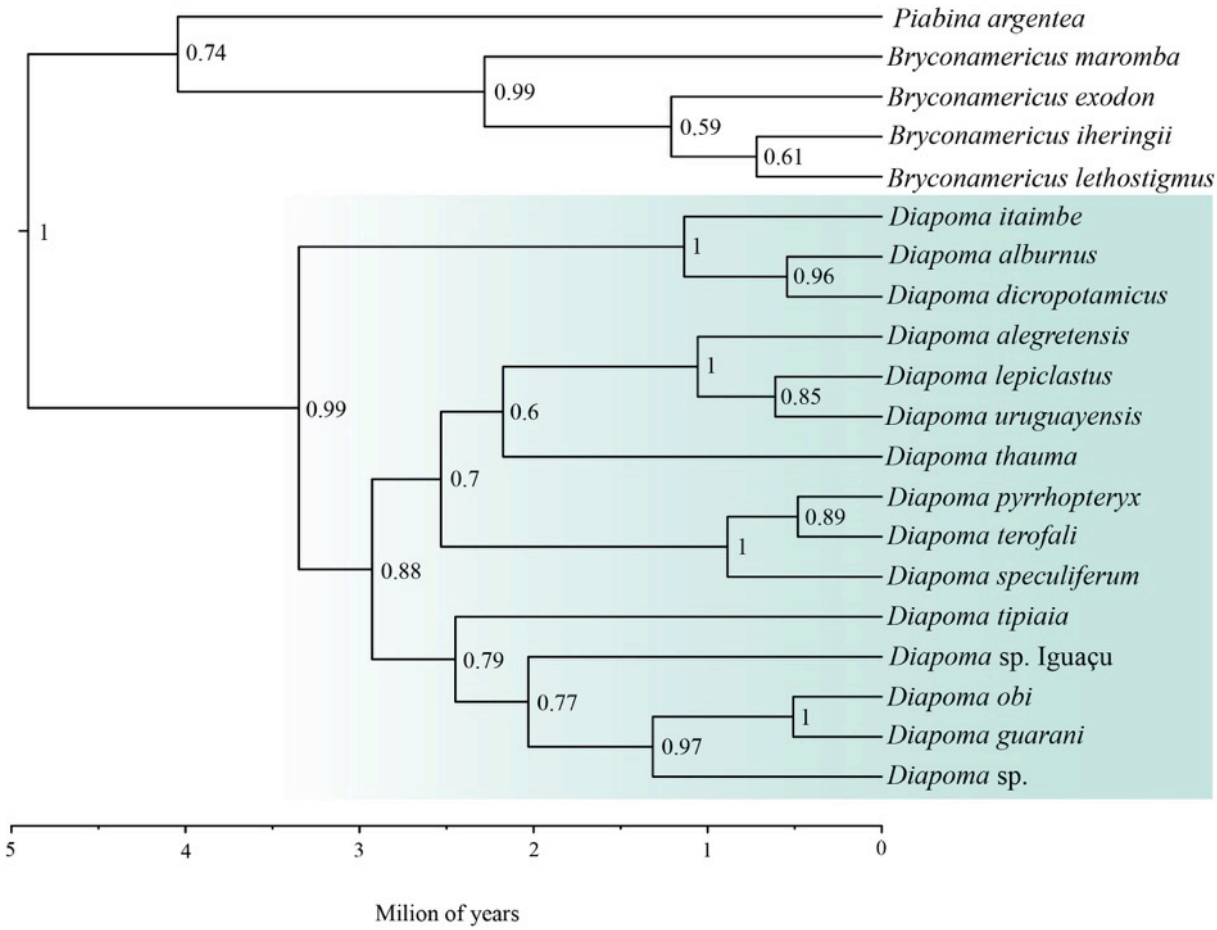
2.000/13
4.000/94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Bryconamericus exodon
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Bryconamericus lethostigmus
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Diapoma dicropotamicus
Diapoma alegretensis
Diapoma lepiclastus
Diapoma uruguayensis
Diapoma thauma
Diapoma pyrrhopteryx
Diapoma terofali
Diapoma speculiferum
Diapoma tipiaia
Diapoma sp. Iguaçu
Diapoma obi
Diapoma guarani
Diapoma sp.
0.74
0.99
0.59
0.61
1
1
0.96
1
0.85
0.99
0.6
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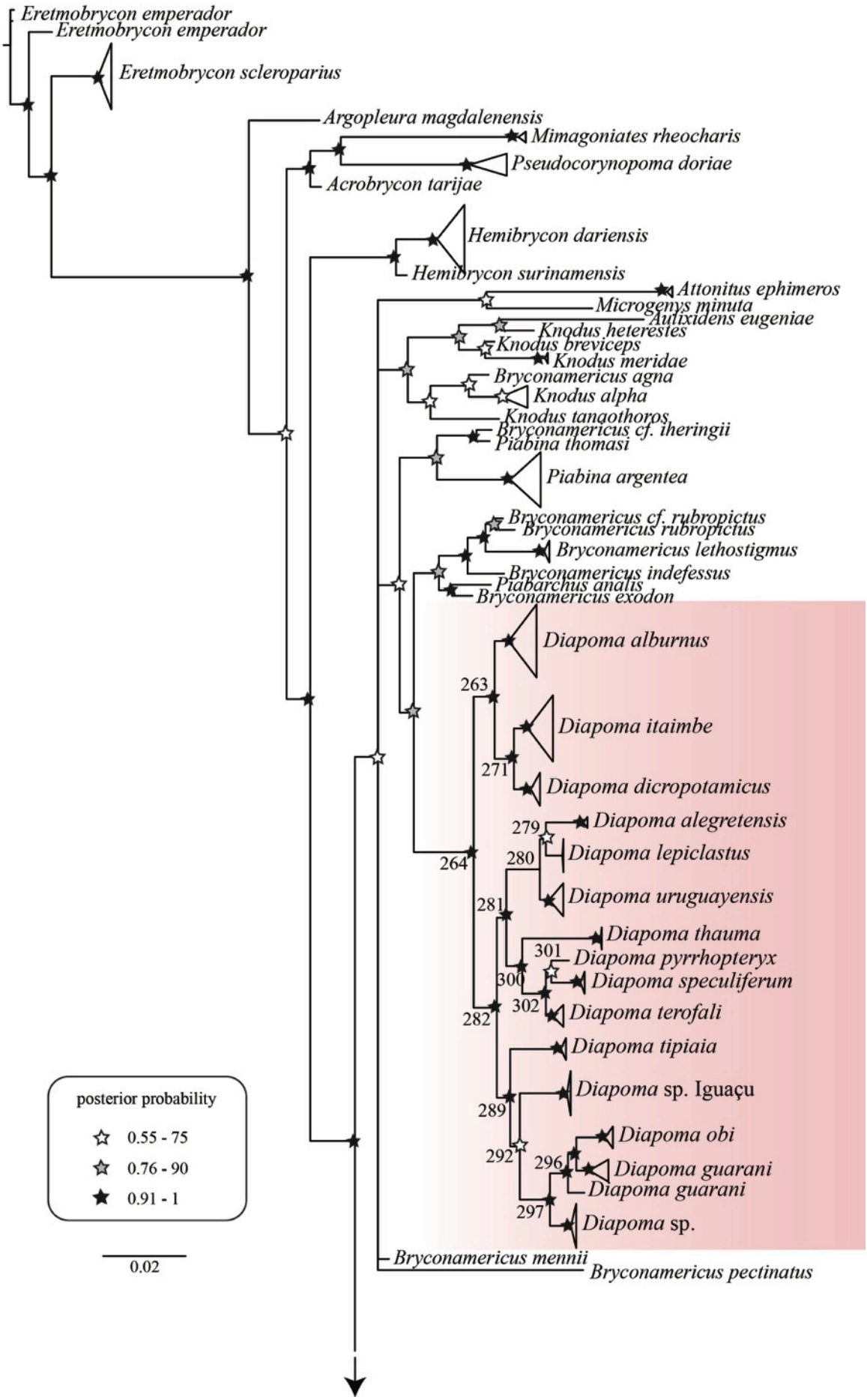
Milion of years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Eretmobrycon emperador
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Acrobrycon tarijae
Hemibrycon dariensis
Hemibrycon surinamensis
Attonitus ephimeros
Microgenys minuta
Autixidens eugeniae
Knodus heterestes
Knodus breviceps
Knodus meridae
Bryconamericus agna
Knodus alpha
Knodus tanqothoros
Bryconamericus cf. iheringii
Piabina thomasi
Piabina argentea
Bryconamericus cf. rubropictus
Bryconamericus rubropictus
Bryconamericus lethostigmus
Bryconamericus indefessus
Piabarchus analis
Bryconamericus exodon
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Diapoma itaimbe
271
Diapoma dicropotamicus
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Diapoma alegretensis
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Diapoma lepiclastus
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Diapoma thauma
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Diapoma speculiferum
302
282
Diapoma terofali
Diapoma tipiaia
Diapoma sp. Iguaçu
289
Diapoma obi
292
296
Diapoma guarani
Diapoma guarani
297
Diapoma sp.
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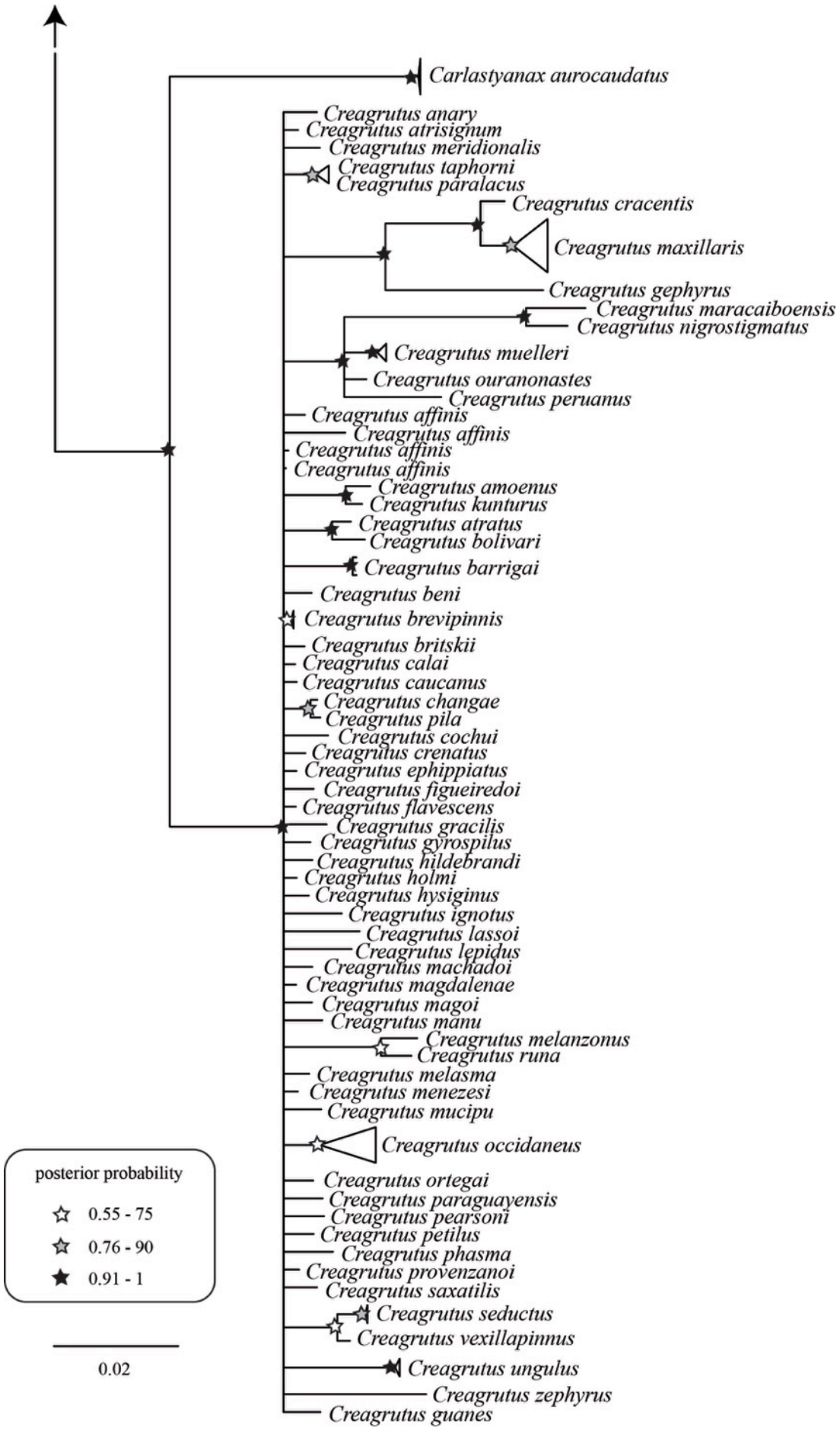
Bryconamericus pectinatus
posterior probability
0.55 - 75
0.76 - 90
0.91 - 1
0.02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Creagrutus muelleri
Creagrutus ouranonastes
Creagrutus peruanus
Creagrutus affinis
Creagrutus affinis
Creagrutus affinis
Creagrutus affinis
Creagrutus amoenus
Creagrutus kunturus
Creagrutus atratus
Creagrutus bolivari
Creagrutus barrigai
Creagrutus beni
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Creagrutus pila
Creagrutus cochui
Creagrutus crenatus
Creagrutus ephippiatus
Creagrutus figueiredoi
Creagrutus flavescens
Creagrutus gracilis
Creagrutus gyrospilus
Creagrutus hildebrandi
Creagrutus holmi
Creagrutus hysiginus
Creagrutus ignotus
Creagrutus lassoi
Creagrutus lepidus
Creagrutus machadoi
Creagrutus magdalenae
Creagrutus magoi
Creagrutus manu
Creagrutus melanzonus
Creagrutus runa
Creagrutus melasma
Creagrutus menezesi
Creagrutus mucipu
Creagrutus occidaneus
Creagrutus ortegai
Creagrutus paraguayensis
Creagrutus pearsoni
Creagrutus petilus
Creagrutus phasma
Creagrutus provenzanoi
Creagrutus saxatilis
Creagrutus seductus
Creagrutus vexillapinnus
Creagrutus ungulus
Creagrutus zephyrus
Creagrutus guanes
posterior probability
0.55 - 75
0.76 - 90
0.91 - 1
0.02

Supporting Information

Appendix S1 Complete list of the analysed discrete morphological characters (Mirande, 2010. 2013).

1. Posterior laminar expansion of epiphyseal bar (0) absent (1) present.

2. Ventral longitudinal lamellae of basioccipital (0) falling short of posterior border of basioccipital (1) reaching posterior border of cranium.

3. Ventral projection of lagenar capsule (0) not extending to basioccipital-parasphenoid articulation (1) extending ventrally to basioccipital-parasphenoid articulation.

4. Epioccipital bridge over posttemporal fossa (0) absent (1) present.

5. Form of epioccipital bridge (0) cylindrical or vertically expanded in transverse section (1) depressed in its middle region.

6. Anterior articulation of epioccipital bridge (0) with both parietal and prootic (1) only with parietal.

7. Posteriorly-oriented epioccipital spine (0) present (1) absent.

8. Ventromedial opening of posttemporal fossa (0) absent (1) present.

9. Position of ventromedial opening of posttemporal fossa (0) between epioccipital and exoccipital (1) bordered entirely by epioccipital.

10. Length of sphenotic spine (0) not extending ventrally to sphenotic-hyomandibular articulation (1) extending ventrally to sphenotic-hyomandibular articulation.

11. Position of sphenotic spine relative to hyomandibula (0) rather aligned with anterior margin of hyomandibula (1) displaced anteriorly relative to anterior margin of hyomandibula.

12. Position of sphenotic spine relative to the orbit (0) bordering orbit posteriorly and aligned with infraorbitals (1) distinctly posterior to orbital margin.

13. Temporal fossa (0) well developed (1) absent or much reduced.

14. Form of anterior process of lateral ethmoid (0) broad in ventral view, contacting vomer in its entire length (1) slender and separated from vomer.

15. Lateral opening between ventral diverging lamellae of mesethmoid and anterior process of lateral ethmoid (0) broad (1) small, ovate and partially occluded by mesethmoid and vomer.

16. Dorsal margin of lateral ethmoids (0) aligned (1) oblique in dorsal view, converging in an anterior angle.

17. Articulation between medial region of lateral ethmoid and frontal or mesethmoid (0) absent (1) extensive articulation of entire lateral ethmoid dorsal margin.

18. Subtemporal fossa (0) medially extended to middle exoccipital (1) restricted to pterotic and prootic.

19. Ascending process on posterodorsal angle of exoccipital directed to neural complex of Weberian apparatus (0) absent (1) present.
20. Anterior extension of frontal (0) reaching posterior margin of nasal opening (1) extending between nasals and reaching middle length of nasal.
21. Contact between frontals anteriorly to frontal fontanel (0) absent (1) present.
22. Frontal fontanel (0) present (1) totally occluded by frontals.
23. Relative size of frontal and parietal fontanels (0) length of frontal fontanel up to 2/3 length of parietal fontanel (1) length of frontal fontanel 3/4 or more of parietal fontanel.
24. Dilator fossa on lateral surface of frontal (0) absent (1) present.
25. Anterior end of mesethmoid (0) trifurcate, with processes inserted into premaxillary depressions (1) not trifurcate, with a triangular anterior spine.
26. Ventral projection of mesethmoid spine, forming a keel between premaxillae (0) absent (1) present.
27. Form of mesethmoid spine (0) long, extending between premaxillae (1) relatively short, with premaxillae articulating anteriorly.
28. Posterior portion of mesethmoid spine (0) relatively slender (1) as broad as lateral wings of mesethmoid.
29. Lateral wings of mesethmoid (0) present (1) absent.
30. Ventral diverging lamellae of mesethmoid (0) absent (1) present.
31. Anterior convergence of ventral diverging lamellae with nasal septum of mesethmoid (0) absent, or confluent near anterior end of nasal septum (1) confluent at posterior end of nasal septum.
32. Nasal septum of mesethmoid (0) single longitudinal lamella (1) two parallel lamellae.
33. Nasal (0) present (1) absent.
34. Bony lamellae bordering sensory canal of nasal (0) absent or more slender than tubular region (1) wider at some point than tubular region.
35. Synchondral articulation between lateral ethmoid and anterodorsal border of orbitosphenoid (0) present (1) absent, with orbitosphenoid distant from lateral ethmoid.
36. Lateral bony coverage of olfactory nerve (0) absent (1) covered by posterior expansion of lateral ethmoid (2) covered by an anterior tubular projection of orbitosphenoid (3) covered by orbitosphenoid and lateral ethmoid.
37. Form of orbitosphenoid (0) slender, relatively small and separate from parasphenoid (1) massive, almost reaching parasphenoid ventrally.
38. Distance between posterodorsal margin of ethmoid cartilage and lateral ethmoids (0)

contacting, or almost contacting, lateral ethmoids (1) distant from lateral ethmoids.
39. Opening between orbitosphenoid and pterosphenoid (0) present, rounded or ovate, usually margined by frontal dorsally (1) absent.
40. Anterior paired projections of parasphenoid (0) absent (1) present.
41. Parietal fontanel (0) present in adults (1) absent in adults.
42. Trigemino-facialis foramen (0) broad, largely limited by sphenotic dorsally (1) narrow, as a cleft with sphenotic almost excluded from margin.
43. Large foramen on pterosphenoid (0) absent (1) present, well developed.
44. Small foramen near posterior margin of pterosphenoid (0) absent, or not pierced by nerves (1) present, pierced by a branch of supraorbital nerve.
45. Dorsal process of pterotic where tendon from epaxial musculature attach (0) absent (1) present, projecting dorsally from tube for semicircular canal.
46. Relative length of pterotic spine (0) projected more posteriorly than hyomandibular ligament (1) restricted to attachment region of hyomandibular ligament.
47. Rhinosphenoid (0) absent (1) present.
48. Dorsal expansion of rhinosphenoid (0) absent or cartilaginous (1) present and forming a bony wall between olfactory nerves.
49. Posterior extension of rhinosphenoid cartilage (0) projected only to middle horizontal length of orbitosphenoid (1) extended to articulation orbitosphenoid-pterosphenoid.
50. Ventral border of rhinosphenoid (0) distinctly separate from parasphenoid (1) almost contacting parasphenoid.
51. Anterior margin of supraoccipital (0) situated completely behind vertical through posterior orbit (1) situated anterior to vertical through posterior orbital margin.
52. Length of supraoccipital spine (0) extends dorsal of entire neural complex of Weberian apparatus (1) extends dorsal of approximately one half of neural complex.
53. Length of supraoccipital spine (0) extends posteriorly to, at least, middle length of neural complex (1) extends only to anterior limit of neural complex.
54. Dorsolateral processes of vomer (0) absent (1) present.
55. Antorbital (0) present (1) absent or fused with first infraorbital.
56. Position of antorbital relative to lateral ethmoid in lateral view (0) antorbital entirely anterior to lateral ethmoid (1) antorbital overlapping lateral ethmoid.
57. Relative position of anterior margin of antorbital and first infraorbital (0) antorbital either aligned or anterior to first infraorbital (1) anterior margin of antorbital posterior to first infraorbital.

58. Bony lamellae bordering laterosensory canal of first infraorbital (0) present (1) absent.
59. Extent of expansion of first infraorbital lateral to maxilla (0) covering less than one half length of maxilla (1) covering most of maxilla.
60. Lateral overlap of first infraorbital by anterior margin of second infraorbital (0) absent (1) present.
61. Overlap of maxilla by second infraorbital (0) absent (1) present.
62. Articulation between second and third infraorbitals (0) vertical (1) anteroventrally angled (2) posteroventrally angled.
63. Anterior region of third infraorbital (0) not much expanded relative to posterior region of second one (1) abruptly expanded relative to posterior region of second one.
64. Ventral extent of third infraorbital (0) not reaching horizontal arm of preopercle, at least anteriorly; (1) reaching horizontal arm of preopercle.
65. Posterior extent of third infraorbital (0) covering angle of preopercle (1) reduced, angle of preopercle covered partially by fourth one.
66. Fourth infraorbital (0) present, well developed (1) absent or reduced and bordered by third and fifth ones.
67. Form of fourth infraorbital (0) square or more developed longitudinally than dorsoventrally (1) longer dorsoventrally than longitudinally.
68. Posterior dorsoventral expansion of fourth infraorbital (0) absent (0) present.
69. Lateral coverage of dilator fossa by sixth infraorbital (0) almost complete, at least in its ventral border (1) leaving a conspicuous naked area in anterior region of fossa.
70. Supraorbital (0) present (1) absent.
71. Contact between supraorbital and sixth infraorbital (0) absent (1) present.
72. Laterosensory canal in antorbital (0) absent (1) present.
73. Laterosensory canal of first infraorbital (0) present (1) absent.
74. Branching of laterosensory canals of fourth or fifth infraorbitals (0) absent (1) present.
75. Direction of posterior branch of laterosensory canal of fourth or fifth infraorbital (0) to a pore on preopercle near hyomandibular condyle (1) to a pore conspicuously ventral to hyomandibular condyle.
76. Laterosensory canal of sixth infraorbital (0) not branched (1) branched.
77. Position of opening on neurocranium communicating with laterosensory canal of sixth infraorbital (0) between frontal and pterotic (1) in frontal.
78. Position of opening on neurocranium communicating with sixth infraorbital laterosensory canal (0) lateral to or slightly anterior to vertical semicircular canal (1) distinctly anterior to

vertical semicircular canal.
79. Length of laterosensory canal of dentary (0) piercing almost entire length of dentary (1) reduced or absent.
80. Pores of laterosensory canal of lower jaw (0) six or less (1) seven or more.
81. Lateral surface of vertical canal of preopercle (0) canal uncovered, posterior to musculature and infraorbitals (1) covered by musculature and/or infraorbitals.
82. Dorsal end of laterosensory canal of preopercle and suprapreopercle (0) not overlapping anterodorsal process of opercle (1) overlapping anterodorsal process of opercle.
83. Anterior region of laterosensory canal of frontal (0) contained completely on frontal (1) opens into a chamber anteriorly.
84. Epiphyseal branch of supraorbital canal (0) present (1) absent.
85. Epiphyseal branch of corresponding supraorbital canals (0) both aligned with epiphyseal bar (1) oriented obliquely, opening posteriorly to epiphyseal bar.
86. Opening of epiphyseal laterosensory canals (0) along margin of cranial fontanel (1) canals continue dorsomedially in soft tissue (2) a single medial pore.
87. Laterosensory canal on sphenotic (0) absent (1) present.
88. Posterior branch of posttemporal laterosensory canal (0) present (1) absent.
89. Form of lateral line (0) approximately straight (1) curved ventrally in abdominal region.
90. Degree of ventral curvature of lateral line (0) straight or only slightly curved (1) distinctly curved and ventrally situated.
91. Lateral line (0) complete (1) interrupted.
92. Canal of lateral line on caudal-fin membrane (0) absent (1) present.
93. Length of caudal-fin canal of lateral line (0) reaching only half of caudal-fin length (1) almost reaching posterior margin of caudal fin.
94. Anterior end of ascending process of maxilla (0) with conspicuous notch (1) pointed or rounded.
95. Ventral margin of toothed region of maxilla (1) approximately straight (1) strongly concave.
96. Margins of toothed region of maxilla (0) roughly parallel (1) dorsally divergent.
97. Expansion of lamellar portion of maxilla just posterior to toothed region (0) absent or not pronounced (1) very pronounced.
98. Tubules for passage of blood vessels on lamellar portion of maxilla (0) a single tubule, parallel to dorsal margin of maxilla (1) with anterior branch running parallel to anterior margin of maxilla (2) anastomosed tubules.

99. Posterior extent of maxilla (0) not reaching second infraorbital (1) reaching second infraorbital.
100. Length of maxilla relative to dentary (0) maxilla reaching posterior end of Meckelian cartilage (1) maxilla not reaching posterior end of Meckelian cartilage.
101. Ontogenetic lengthening of maxilla (0) absent (1) present.
102. Dorsal projection of maxilla (0) not overlaps second infraorbital (1) overlaps second infraorbital.
103. Interdigitations between premaxillae (0) present (1) absent.
104. Length of ascending process of premaxilla (0) reaching at least one-third of length of nasal (1) reaching just anterior end of nasal.
105. Alignment of ascending process of premaxilla (0) aligned with medial margin of nasal (1) medially shifted and separated from nasal.
106. Form of posterolateral portion of premaxilla (0) with notch (1) with pedicle expanded laterally to maxilla.
107. Lateral ridge of anguloarticular (0) absent (1) present.
108. Horizontal process of anguloarticular (0) laterally covered by dentary only anteriorly (1) broadly covered by dentary to tip of Meckelian cartilage.
109. Ventral margin of horizontal process of anguloarticular (0) posteroventrally angled relative to dentary laterosensory canal (1) perpendicular to laterosensory canal of dentary.
110. Position of coronomeckelian (0) situated mainly lateral to Meckelian cartilage (1) situated mainly dorsal to Meckelian cartilage.
111. Interdigitations between dentaries (0) absent (1) present.
112. Form of interdigitations between dentaries (0) simple bony lamellae (1) undulate lamellae.
113. Form and dentition of anterior region of dentary (0) toothed and not depressed anteriorly (1) edentulous and much depressed anteriorly.
114. Medial anteroventral notch of dentary (0) absent (1) present.
115. Medial process of dentary bordering Meckelian cartilage dorsally and medially (0) absent (1) present.
116. Bony lamella covering dentary foramen laterally (0) absent (1) present.
117. Longitudinal ridge covering laterosensory pores of dentary (0) absent (1) present.
118. Morphology of premaxillary, maxillary, and dentary teeth (0) all teeth conical, caniniform or mamilliform (1) some teeth multicuspidate or molariform (2) all teeth bicuspidate.

119. Premaxillary, maxillary, and dentary teeth (0) not pedunculate, or pedunculate only in some of these bones (1) pedunculate and uniformly shaped.
120. Mamilliform teeth outside mouth (0) absent (1) present.
121. A pair of large conical teeth in premaxilla (0) absent (1) present.
122. Number of rows of premaxillary teeth (0) one (1) two or three.
123. Number of rows of premaxillary teeth (0) one or two (1) three.
124. Alignment of teeth on anterior premaxillary row (0) aligned (1) not aligned, with one or two teeth situated anteriorly.
125. Cusps of teeth on outer premaxillary row (0) one to three cusps (1) five or more cusps.
126. Cusps of teeth on inner premaxillary row (0) molariform (1) aligned in straight series or with anteriorly concave pattern (2) with anteriorly concave pattern plus anterior cusps.
127. Alignment of cusps of medial teeth on inner premaxillary row (0) forming anteriorly concave semicircle from ventral view (1) forming shallow arch or aligned in straight series.
128. Form of teeth of inner premaxillary tooth row (0) with cusps forming anteriorly concave arch (1) with cusps aligned in straight series.
129. Number of teeth in inner premaxillary row (0) four or fewer (1) five or more.
130. Number of teeth in inner premaxillary row (0) seven or fewer (1) eight or more.
131. Polymorphism of teeth on inner premaxillary row (0) absent (1) present, with two medial teeth somewhat larger.
132. Number of replacement tooth rows on premaxilla (0) one (1) two or more.
133. Fossa for inner row of replacement premaxillary teeth (0) absent (1) present.
134. Maxillary teeth (0) absent (1) present.
135. Number of maxillary teeth (0) only one, or absent (1) two or more.
136. Number of maxillary teeth (0) up to three (1) four or more.
137. Extent of implantation of teeth along maxilla (0) not reaching middle of maxillary lamella (1) extending across almost entire maxillary lamella.
138. Number of cusps of anterior maxillary teeth (0) conical, a single cusp (1) three or more cusps.
139. Number of cusps of anterior maxillary teeth (0) up to three (1) five or more cusps.
140. Ontogenetic acquisition of conical teeth on maxilla (0) absent (1) present.
141. Orientation of anterior dentary teeth (0) oriented dorsally or anterodorsally (1) oriented anteriorly, almost parallel to main axis of dentary.
142. Size and number of anterior dentary teeth (0) four or five relatively broad teeth at front of dentary (1) eight or more small and slender teeth at front of dentary.

143. Inner row of dentary teeth (0) present (1) absent.
144. Symphyseal dentary tooth (0) absent (1) present.
145. Articulation between dentary teeth (0) absent (1) present with associated processes and fossae.
146. Position of anterior teeth of dentary (0) along margin of dentary (1) internally situated with dentary forming anterior ridge.
147. Separation between posterior dentary teeth (0) less than width of these teeth (1) more than width of these teeth.
148. Abrupt decrease in size of dentary teeth (0) absent (1) present.
149. Foramen on articular condyle of quadrate (0) absent (1) present.
150. Form of quadrate (0) with ventral portion longer than anterodorsal region (1) with anterodorsal portion equal or longer than ventral region.
151. Posterior extent of ventral process of quadrate (0) reaching vertical through posterior margin of symplectic (1) falling short of posterior margin of symplectic.
152. Longitudinal ridge in quadrate bordering *adductor mandibulae* muscle ventrally and, to some degree laterally (0) absent (1) present.
153. Articulation between quadrate and anguloarticular (0) anterior to or at vertical through lateral ethmoid (1) posterior to lateral ethmoid.
154. Articulation between quadrate and anguloarticular (0) anterior to or at vertical through middle eye (1) posterior to middle eye.
155. Articulation between ventral margin of metapterygoid and posterodorsal margin of quadrate (0) absent (1) present.
156. Shape of ectopterygoid (0) elongate (1) triangular and much broadened anteriorly (2) approximately square.
157. Form of anterior portion of ectopterygoid (0) broad and broadly articulating with palatine (1) slender and articulating only to lateral margin of palatine.
158. Dorsal process of ectopterygoid oriented towards lateral ethmoid (0) absent (1) present.
159. Ectopterygoid teeth row (0) absent (1) present.
160. Patch of ectopterygoid teeth (0) absent (1) present.
161. Position of longitudinal cartilage dorsal to ectopterygoid (0) bordered medially by mesopterygoid (1) displaced laterally and separated from mesopterygoid.
162. Contact between ectopterygoid and anterodorsal region of quadrate (0) present (1) absent.
163. Anterior extension of interopercle (0) extending anteriorly beyond tip of horizontal arm

of preopercle (1) not extending anteriorly beyond anterior tip of preopercle.
164. Abrupt posterior expansion of interopercle (0) absent (1) present.
165. Mesopterygoid teeth (0) absent (1) present.
166. Anterodorsal lobe of metapterygoid oriented towards mesopterygoid (0) absent or small and dorsally oriented (1) present, conspicuous and anteriorly oriented.
167. Shape of metapterygoid-quadrate fenestra (0) rounded or ovate, anteriorly limited by quadrate (1) anteriorly collapsed by metapterygoid and quadrate.
168. Foramen in posterior region of metapterygoid (0) absent (1) present, encircled by metapterygoid or bordered by cartilage (2) an incomplete arch, bordered posteriorly by hyomandibula.
169. Posterior directed radial striae from articular region of opercle (0) absent (1) present.
170. Length of medial bony ridge of opercle (0) 60% or greater than opercular length (1) less than 50% of opercular length.
171. Ethmopalatine cartilage (0) absent or reduced in size (1) present and conspicuous.
172. Relative length of palatine (0) approximately one-half length of ectopterygoid, or less (1) distinctly longer than one-half length of ectopterygoid.
173. Palatine foramen (0) absent or reduced in size (1) present and very conspicuous.
174. Shape of posteroventral corner of preopercle (0) acute (1) rounded.
175. Suprapreopercle (0) fused to preopercle (1) autogenous, separated from preopercle.
176. Bony lamellae bordering laterosensory canal of suprapreopercle (0) absent (1) present.
177. Anterior projection of anterior ceratohyal articulating laterally with hypohyals (0) absent or much reduced (1) present and achieving half length of hypohyals.
178. Hyoid artery (0) completely contained within anterior ceratohyal (1) emerging from anterior ceratohyal.
179. Ventral margin of anterior ceratohyal (0) smooth and without notches (1) with notches for articulation of branchiostegal rays.
180. Number of notches along ventral border of anterior ceratohyal (0) zero to two (1) three.
181. Articulation between anterior and posterior ceratohyals (0) synchondral, without bony interdigitations (1) with bony interdigitations.
182. First basibranchial (0) absent or much reduced, not articulating with basihyal (1) well developed and articulating anteriorly with basihyal.
183. Contact between lamella on anterior portion of first basibranchial with lamella on posterior portion of second basibranchial (0) absent (1) present.
184. Bony lamellae between second and third basibranchials (0) absent (1) present.

185. Bony lamella dorsal to fourth basibranchial (0) present (1) absent.
186. Main portion of fourth basibranchial (0) completely cartilaginous (1) ossified.
187. Teeth on lamella dorsal to fourth basibranchial (0) absent (1) present.
188. Cartilages anterior to basihyal (0) one or two blocks of cartilage, but anterior block much smaller (1) two well developed blocks of cartilage.
189. Edentulous basihyal lamella (0) absent (1) present.
190. Anterior development of basihyal (0) slightly surpassing anterior margin of hypohyals (1) broadly extending beyond anterior margin of hypohyals.
191. Form of anterior expansion of basihyal (0) slender, with anterior margin less than two-thirds of its length (1) expanded anterior margin, with two-thirds or more of its length.
192. Rows of gill rakers on first ceratobranchial (0) one (1) two.
193. Rows of gill rakers on second ceratobranchial (0) one (1) two.
194. Rows of gill rakers on third and fourth ceratobranchials (0) one (1) two.
195. Number of gill rakers on first hypobranchial and ceratobranchial (0) 16 or more (1) 15 or fewer.
196. Number of gill rakers on first hypobranchial and ceratobranchial (0) 11 or more (1) ten or fewer.
197. Shape of first ceratobranchial gill rakers (0) pointed and not anteroposteriorly compressed (1) laminar, much compressed perpendicular to ceratobranchial (2) short, broad and strongly denticulate.
198. Form of anterior gill rakers on first ceratobranchial (0) not fused (1) with fused bases forming plates.
199. Lateral base of gill rakers on first ceratobranchial (0) slender (1) broad and laminar at least on anteriormost gill rakers.
200. Form and degree of ossification of first ceratobranchial gill rakers (0) laminar and not ossified distally (1) rather thick and completely ossified distal region.
201. Denticles on gill rakers (0) present (1) absent.
202. Distribution of denticles on gill rakers (0) restricted to margins, or absent (1) along entire surface of gill rakers.
203. Rows of gill rakers on first epibranchial (0) one (1) two.
204. Shape of dentigerous plate of fifth ceratobranchial (0) rounded, with posterior notch (1) elongated, without posterior notch.
205. Teeth on fifth ceratobranchial (0) present (1) absent.
206. Teeth on third pharyngobranchial (0) present (1) absent.

207. Teeth on fourth pharyngobranchial (0) present (1) absent.
208. Teeth on fifth pharyngobranchial (0) present (1) absent.
209. Contact between fourth and fifth pharyngobranchial dentigerous plates (0) absent (1) present.
210. Interhyal (0) present (1) absent.
211. Length of interhyal (0) shorter than one-third of symplectic length (1) equal to or longer than one-half of symplectic length.
212. Number of branchiostegal rays (0) three (1) four or five.
213. Number of branchiostegal rays (0) three or four (1) five.
214. Anterior portions of branchiostegal rays (0) broad near their articulation with ceratohyals (1) slender near their articulation with ceratohyals.
215. Attachment of first branchiostegal ray (0) on proximal one-half length of anterior ceratohyal (1) posterior to one-half length of anterior ceratohyal.
216. Distance between attachment site of first and second branchiostegal rays (0) equal or shorter than distance between second and third rays (1) longer than distance between second and third rays.
217. Number of branchiostegal rays attached to posterior ceratohyal (0) one (1) two.
218. Form and articulation of neural pedicle of third vertebra (0) pedicle articulating synchondrally with neural comlex (1) pedicle without an articular surface with neural complex.
219. Development of transverse process of neural arch of third vertebra (0) not reaching anterior margin of tripus (1) well developed and extending beyond anterior margin of tripus.
220. Ascending process of neural pedicle of third vertebra (0) absent (1) present.
221. Dorsal development of dorsal process of neural pedicle of third vertebra (0) not broadly overlapping neural complex (1) broadly overlapping neural complex.
222. Neural arch and vertebral centrum of fourth vertebra (0) not fused and with autogenous fourth neural arch (1) fused.
223. Anteriorly directed spine at base of first rib (0) absent (1) present.
224. Laminar bony ridge on dorsal margin of abdominal ribs (0) absent (1) present.
225. Abdominal ribs on anterior caudal vertebrae (0) absent (1) present, associated to first and second caudal vertebrae.
226. Relative number of precaudal vertebrae (0) exceeding caudal vertebrae in two or more elements (1) equal or less numerous than caudal vertebrae.
227. Total number of vertebrae (0) 40 or fewer (1) 41 or more.

228. Total number of transitional vertebrae (0) four or more (1) three or fewer.
229. Transitional vertebrae with haemal canal (0) present (1) absent.
230. Margin of first pectoral ray in adult specimens (0) not serrated (1) conspicuously serrated.
231. Base of second pectoral ray (0) large and partially medially overlapping base of first pectoral ray (1) similar in form and size to base of posterior rays.
232. Anterior margin of cleithrum (0) slightly sinuous (1) with anterior pointed projection.
233. Form of posterior margin of cleithrum (0) convex or slightly sinuous just dorsal to pectoral-fin insertion (1) with notch just anterior to pectoral-fin insertion.
234. Posterior margin of cleithrum (0) without concavity ventral to first postcleithrum (1) with concavity ventral to first postcleithrum.
235. Posterior margin of cleithrum (0) with concavity poorly pronounced or lacking (1) with markedly concave margin, almost forming straight angle.
236. Medial laminar expansion at dorsal tip of cleithrum (0) absent (1) present.
237. Dorsal development of cleithrum (0) much extended dorsally to mesocoracoid (1) ending in a position just dorsal of tip of mesocoracoid.
238. Development of medial lamella of coracoid (0) not expanded (1) expanded as a keel.
239. Bony ridge of coracoid between base of mesocoracoid and ventral margin of interosseous space (0) absent (1) present.
240. Anterior extension of coracoid ventral lamella (0) reaching cleithrum (1) not reaching cleithrum.
241. Ventral extension of coracoid lamella (0) reaching ventral margin of cleithrum (1) falling short of ventral margin of cleithrum.
242. Anterior limit of interosseous space (0) formed by dorsal margins of coracoid and cleithrum (1) formed by coracoid and an oblique ridge of cleithrum.
243. Coracoid foramen (0) absent or reduced to small pore (1) well developed.
244. Process of scapula forming anterior border of scapular foramen (0) present (1) absent.
245. Articulation between ventral process of mesocoracoid and dorsal margin of scapula (0) absent or small (1) present and broad.
246. Ventral articulation of mesocoracoid (0) anteriorly with coracoid and posteriorly with scapula (1) only with coracoid.
247. First postcleithrum (0) present (1) absent.
248. Second postcleithrum (0) present (1) absent.
249. Third postcleithrum (0) present (1) absent.

250. Form of third postcleithrum (0) slender, without associated lamella (1) with a posterior lamella.
251. Dorsal development of third postcleithrum (0) projects dorsally to posterior region of scapula (1) not projects dorsally to posterior region of scapula.
252. Position of ventral margin of posttemporal (0) anterior to lateral margin of epioccipital (1) lateral or posterior to lateral margin of epioccipital.
253. Position of ventral end of posttemporal (0) anterior or lateral to lateral margin of epioccipital (1) posterior to lateral margin of epioccipital.
254. Ventral exit of laterosensory canal of supracleithrum (0) medial, covered by posterior lamella of supracleithrum (1) posterior, ventral to lamella of supracleithrum.
255. Fusion between posttemporal and supracleithrum (0) absent (1) present.
256. First pelvic-fin ray (0) not branched (1) branched.
257. Relative length of first pelvic-fin ray of adult males (0) not extending beyond margin of other rays (1) extending beyond margin of other rays.
258. Number of branched pelvic-fin rays (0) six or less (1) seven or more.
259. Number of branched pelvic-fin rays (0) seven or less (1) eight or more.
260. Pelvic bone (0) not bifurcate anteriorly (1) bifurcate with conspicuous notch.
261. Articulation between pelvic bones (0) through ligaments (1) with bony interdigitations between ischiatic processes.
262. Anterior extension of pelvic-bone along main axis (0) not projecting anterior of lateral and medial lamellae (1) projecting anterior of lateral and medial lamellae of pelvic bone.
263. Anterior tip of pelvic bone (0) rounded and capped by a small cartilage (1) pointed and without cartilage.
264. Dorsal longitudinal ridge on medial lamella of pelvic bone (0) present (1) absent.
265. Relative position of dorsal-fin anterior insertion (0) anterior to or at vertical through pelvic-fin origin (1) posterior to vertical through pelvic-fin origin.
266. Dorsal-fin rays articulating with first dorsal pterygiophore (0) two (1) three or four.
267. Anteriorly oriented spine formed by first dorsal-fin ray (0) absent (1) present.
268. Anterior rays of dorsal fin of adult males (0) not elongate (1) elongate and reaching posteriorly close to adipose fin.
269. Last unbranched dorsal-fin ray of adult males (0) approximately as long as first branched ray (1) distinctly longer than first branched ray and filamentous.
270. Number of branched-rays on dorsal-fin (0) eight or fewer (1) nine or more.
271. Relative length of anterior dorsal-fin rays (0) not reaching tip of posterior rays when

adpressed (1) reaching tip of posterior rays when adpressed.
272. Number of dorsal-fin rays on last pterygiophore (0) one (1) two, adnate.
273. Dorsal myorhabdoi (0) absent (1) present.
274. Position of anteriormost epineurals (0) lateral to fourth or fifth vertebrae (1) reaching to cranium.
275. Predorsal spine formed by first dorsal pterygiophore (0) absent (1) present.
276. Number of dorsal pterygiophores (0) nine or fewer (1) ten or more.
277. Number of dorsal pterygiophores (0) 10 or fewer (1) 11 or more.
278. Number of dorsal pterygiophores (0) 11 or fewer (1) 12 or more.
279. Supraneural anterior to neural spine of fourth vertebra (0) absent or small (1) present and vertically elongate.
280. Number of supraneurals (0) four or fewer (1) five or more.
281. Number of supraneurals (0) seven or fewer (1) eight or more.
282. Bony lamellae associated with supraneurals (0) absent or small (1) wider than primary axis of supraneurals.
283. Position of last supraneural (0) two or fewer vertebrae in front of first dorsal pterygiophore (1) more than two vertebrae in front of first dorsal pterygiophore.
284. Anal-fin position (0) posterior or almost posterior to vertical through last dorsal-fin ray (1) extended anteriorly ventral to dorsal fin.
285. Number of unbranched anal-fin rays (0) three or fewer (1) four or more.
286. Number of branched anal-fin rays (0) ten or fewer (1) 11 or more.
287. Number of branched anal-fin rays (0) 17 or fewer (1) 18 or more.
288. Number of branched anal-fin rays (0) 24 or fewer (1) 25 or more.
289. Number of branched anal-fin rays (0) 34 or fewer (1) 35 or more.
290. Form and length of anterior anal-fin rays (0) similar to posterior rays (1) longer and more compressed laterally than posterior rays.
291. Number of rays on last anal pterygiophore (0) two (1) one.
292. Anterior notch on first anal pterygiophore (0) absent (1) present.
293. Number of anal pterygiophores anterior to first haemal spine (0) three or fewer (1) four or more.
294. Proximal and medial radials of anal fins (0) fused on anterior five pterygiophores (1) fused in most pterygiophores (2) medial radials absent or completely fused with proximal ones.
295. Lateral lamellae on anterior anal pterygiophores (0) absent (1) present.

296. Number of epurals (0) one (1) two or three.
297. Number of epurals (0) one or two (1) three.
298. Fusion of hypural 2 to compound centrum (0) absent (1) present.
299. Fusion between hypurals 1 and 2 (0) absent (1) present.
300. Posterior margin of hypural 3 (0) equal to or narrower than posterior margin of hypural 4 (1) deeper than posterior margin of hypural 4.
301. Ventral procurrent caudal-fin rays of adult males (0) slender (1) laminar.
302. Number of ventral procurrent caudal-fin rays (0) 11 or fewer (1) 12 or more.
303. Ventral procurrent caudal-fin rays of adult males (0) not projecting through musculature and skin of peduncle (1) projecting ventrally through peduncle musculature and skin.
304. Caudal-fin bony stays (0) absent (1) present.
305. Anterior ventral procurrent caudal-fin rays (0) paired, only distally fused (1) fused in laminar medial bones. 306. Uroneurals (0) absent or just one pair (1) two pairs.
307. Bony hooks on fin rays (0) absent (1) present in adult males.
308. Anal-fin bony hooks in adult males of species bearing hooks on fins (0) absent (1) present.
309. Pelvic-fin bony hooks in adult males of species bearing hooks on fins (0) absent (1) present.
310. Pectoral-fin bony hooks in adult males of species bearing hooks on fins (0) absent (1) present.
311. Dorsal-fin bony hooks in adult males of species bearing hooks on fins (0) absent (1) present.
312. Caudal-fin bony hooks in adult males of species bearing hooks on fins (0) absent (1) present.
313. Bony hooks on base of pelvic-fin rays of adult males (0) absent, or in small number compared to on segmented portion (1) as numerous as on segmented portion of rays.
314. Bony hooks on last pelvic-fin ray of adult males (0) absent or reduced in number (1) as numerous as in other rays.
315. Bony hooks on first pelvic-fin ray of adult males (0) absent (1) present.
316. Position of anal-fin bony hooks of adult males (0) paired and ordered laterally or posterolaterally (1) medially positioned and oriented posteriorly (2) asymmetrically disposed and irregularly arranged.
317. Scales (0) cycloid (1) ctenoid (2) spinoid (3) crenate.
318. Anterior margin of scales (0) uniformly curved or slightly undulated (1) with

conspicuous undulations.
319. Circuli on posterior field of scales (0) present (1) absent.
320. Radii on scales (0) absent or reduced in number (1) present and numerous on most scales.
321. Radii oriented towards anterior field of scales (0) present (1) only as longitudinal groove without defined margins (2) absent.
322. Radii of scales (0) not converging at focus (1) converging at focus.
323. Semicircular grooves on posterior field of scales (0) absent (1) present.
324. Scales covering supraoccipital spine (0) absent (1) present and completely covering supraoccipital spine.
325. Median predorsal scales (0) covering entire predorsal region (1) leaving naked area anterior to dorsal fin.
326. Ventral serrae (0) absent (1) present.
327. Scales covering anal-fin base (0) one or two rows of scales covering anal-fin base (1) several rows covering basal third of anal fin.
328. Scales covering caudal-fin lobes (0) covering only their base (1) covering one-third of their length.
329. Ventral division of tendon from *adductor mandibulae* inserted on dentary (0) absent (1) present.
330. Longitudinal position of insertion of *adductor mandibulae* tendon on dentary (0) on vertical through Meckelian cartilage posterior half (1) on vertical through Meckelian cartilage middle or anterior half.
331. Insertion of *adductor mandibulae* tendon on dentary (0) ventral to Meckelian cartilage (1) anterior to Meckelian cartilage (2) on a medial process of dentary.
332. Posterior attachment of section A1 from *adductor mandibulae* (0) principally to vertical arm of preopercle (1) restricted or almost restricted to horizontal arm of preopercle.
333. Attachment of medial tendon of section A1 of *adductor mandibulae* (0) on quadrate near its articulation with preopercle (1) on preopercle posterior to quadrate (2) on preopercle ventral to quadrate.
334. Anterior insertion of A1 section of *adductor mandibulae* (0) on maxilla (1) on coronoid process of dentary.
335. Contact between dorsal margin of *adductor mandibulae* and ventral margin of *dilator operculi* (0) absent (1) present.
336. Anterior extension of *adductor arcus palatini* (0) covering most of dorsal surface of

mesopterygoid (1) covering only half of dorsal surface of mesopterygoid.
337. Posterior region of *levator arcus palatini* (0) limited by *adductor mandibulae* and *adductor arcus palatini* (1) limited by A2 and A3 sections of *adductor mandibulae*.
338. Origin of *dilator operculi* (0) anterior to vertical through posterior margin of eye (1) completely posterior to vertical through posterior margin of eye.
339. Pseudotympanum (0) completely absent (1) absent but with muscles reduction (2) present with muscles absent.
340. Insertion of pterotic aponeurosis (0) on pterotic spine or lateral to horizontal semicircular canal (1) on a lobe situated dorsal to horizontal semicircular canal (2) on pterotic or sphenotic, dorsal to semicircular canal.
341. Humeral spot (0) absent (1) present.
342. Second humeral spot (0) absent (1) present.
343. Dark conspicuous spot on dorsal fin (0) absent (1) present.
344. Horizontal line of chromatophores just dorsal to anal-fin base (0) absent (1) present.
345. Color of caudal-fin lobes (0) symmetrically hyaline, yellowish, reddish, or violaceous (1) ventral lobe orange or reddish and dorsal lobe hyaline (2) ventral lobe dark brown or black and dorsal lobe hyaline (3) both lobes dark brown or black.
346. Diffuse spots on flanks (0) absent (1) present, especially in young specimens.
347. Little spot on each scale of flanks (0) absent (1) present.
348. Dark spot covering the entire depth of caudal-fin base (0) absent (1) present.
349. Ventral union of gill membranes (0) joined anteriorly, but not covering the isthmus (1) joined along length of the isthmus but not attached to isthmus (2) joined to each other and with isthmus.
350. Sclerotic bones (0) single anteroventrally open bone (1) two bones separated by cartilages.
351. Nostrils (0) rounded and divided only by skin fold (1) nostrils distinctly separate.
352. Gill-derived gland on males (0) absent (1) present.
353. Glandular tissue of granular appearence on caudal fin (0) absent in mature males (1) present in mature males.
354. Hypertrophied ventral caudal peduncle squamation (0) absent (1) present, restricted to scales below lateral line.
355. Caudal gland cells consisting of modified mucous cells (0) absent (1) present.
356. Adipose fin (0) present (1) absent.
357. Papillae on tongue (0) not aligned (1) forming longitudinal rows anteriorly.

358. Insemination (0) absent (1) present.
359. Sperm nuclei (0) spherical (1) elongated towards the flagellate axis (2) elongated anteriorly in the opposite direction to the flagellum.
360. Testicle with three partitions (0) absent; (1) present.
361. Number of 2n chromosomes (0) 36 to 42 (1) 46 or more.
362. Number of 2n chromosomes (0) 48 or fewer (1) 50 or more.
363. Number of 2n chromosomes (0) 50 or fewer (1) 52 or more.
364. Number of 2n chromosomes (0) 52 or fewer (1) 54 or more.
365. Number of 2n chromosomes (0) 54 or fewer (1) 58 or more.
366. Form of distal tip of sphenotic spine (0) slender or somewhat expanded (1) notched, limiting *adductor operculi* anterior and dorsally.
367. Form of third dentary tooth (0) similar to remaining teeth of dentary (1) strongly decurved posteriorly.
368. Premaxillary dentition (0) one or more series of similar teeth (1) with a recognizable triad of larger teeth with rounded base.
369. Gap between medial two teeth from anterior premaxillary row (0) distinctly greater than gap between remaining teeth (1) equal to gap between remaining teeth.
370. Position of lateralmost tooth of outer premaxillary row (0) anteriorly displaced relative to remaining teeth. (1) aligned with remaining teeth of row.
371. Anterior process of maxilla (0) slender relative to laminar region (1) as thick as posterior region.
372. Flexion on maxilla posterior to alveolar premaxillary arm (0) absent or not pronounced, maxilla rather straight (1) pronounced.
373. Form and attachment of primordial ligament (0) flat and attaching to ascending process of maxilla (1) rotund and attached to distal half of maxillary lamella.
374. Form and attachment of primordial ligament (0) attached to middle length of maxillary lamella or dorsal to it (1) attached near distal tip of maxilla.
375. Posterior attachment of primordial ligament (0) exclusively to anguloarticular (1) with a division attaching to quadrate.
376. Attachment of maxilla with premaxilla (0) a weak ligament attached to a moderately bifurcated premaxilla (1) a strong ligament attached to a greatly bifurcated premaxilla.
377. Ligament from distal tip of ascending maxillary process to premaxilla (0) absent (1) present.
378. Ligament from middle length of ascending maxillary process to premaxilla (0) absent (1)

present.

379. Number of dentary teeth on anterior row (0) eight or fewer (1) ten or more.

380. Number of cusps of anterior dentary teeth (0) three or fewer (1) five or more.

381. Expansion of dentary lateral to anguloarticular (0) closer to Meckelian cartilage than to articular socket (1) closer to articular socket than to Meckelian cartilage.

382. Form of posterodorsal portion of anguloarticular (0) vertical (1) anterodorsally angled.

383. Posterior development of mesopterygoid (0) overlapping metapterygoid (1) separate from metapterygoid.

384. Medial dorsal ridge of quadrate (0) absent (1) present, covering symplectic from lateral view.

385. Form of fourth infraorbital (0) quadrangular, reaching posterior margin of infraorbitals (1) triangular, excluded from posterior margin of infraorbital series.

386. Parietal branch of laterosensorial system (0) reaching posterior half of parietal (1) absent or reaching only to anterior half of parietal.

387. Cartilage-filled space anterior to scapular foramen (0) present, wider than anterior process of scapula (1) much reduced by expansions of coracoid and cleithrum.

388. Number of scales in lateral series (0) 33 or more (1) 32 or fewer.

389. Post-anal scales (0) one or two scales (1) four or five scales.

390. Midlateral body pigmentation (0) not pigmented or with a continuous lateral stripe (1) with a series of dark spots that eventually coalesce.

391. Number of vertebrae (0) up to 33 (1) 34 or more.

392. Notochondral mineralizations (0) absent (1) present.

393. Rhinosphenoid constitution (0) cartilaginous (1) ossified rhinosphenoid.

394. Posteromedial branch of supraorbital canal (0) opens into frontal (1) opens into parietal.

395. Operculum shape (0) the opercle and subopercle not prolonged (1) operculum prolonged posteriorly consisting of a triangular extension of the posteroventral field of the opercle and a posteriorly broadened posterior region of the subopercle.

396. Intense blue color in mature specimens (0) absent (1) present.

397. Black pigmented adipose fin: (0) absent (1) present

398. Shape of anal fin male (0) anal fin with a concave distal bordes (1) nearly straight distal anal fin margin (2) deeply convex margin.

399. Anal spot (0) absent (1) present.

Appendix S2 Complete discrete morphological data matrix. Polymorphisms are denoted as follows: z=[01]; y=[12] and x=[03].

Argopleura magdalenensis

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0011110z11	1?????000?	1000000001	1010000?0?	010???????

Attonitus ephimeros

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0010100010	0000110000	0100000000	1000011000	0000001010
1000010111	0001100001	1200000001	000011110?	0??0000000
?010000011	0?????000?	100000000z	0010000010?	010???????

Aulixidens eugeniae

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00??0?00??	??????000?	?10z000000	101000z?0?	010???????

Bryconamericus agna

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0001100001	0010000010	1010001010	0100100000	0000z00000
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Knodus alpha

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0001100001	0010000010	1010001011	0100100000	0000000000
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Bryconamericus cf. *iheringii*

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Bryconamericus cf. *rubropictus*

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Bryconamericus mennii

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Bryconamericus rubropictus

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Eretmobrycon scleroparius

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Piabina thomasi

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Hemibrycon dariensis
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Knodus heterestes
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Mimagoniates rheocharis
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Eretmobrycon emperador
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Creagrutus anary
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Creagrutus atrisignum
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Creagrutus meridionalis
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0011000000 0010000010 1000100011 0010000000 1000111000
0101001001 0010000010 1010001010 0100110000 0000z10000
0010100010 0000100000 0100000000 00100z1000 0000001010
10z0010111 0001000001 1200000001 11?01????? 0??0000000
?0??0000?? ??????0011 0111011110 0101100100 010???????

Creagrutus taphorni
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0010100010 0000110000 0100000000 10z000z000 0000001010

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Creagrutus cracentis
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0??0100010 0000100000 0100000000 1000000000 0000001010
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Creagrutus gephyrus
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Creagrutus maracaiboensis
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Creagrutus muelleri
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Creagrutus ouranonastes
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00?0100010 0000110000 0100000000 1000001000 0000001010
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Creagrutus peruanus

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Carlastyanax aurocaudatus

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z011100000 00z0110000 0100000000 z000011100 0000001z10
10000z00?? ???????001 1200000001 100011000? 0??0000000
000000000? ??????0110 0011001011 10010?100? 010???????

Microgenys minuta

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01?1100001 0010000010 1010001010 0100100000 0000110000
0010100010 00001100?? 0100000000 1000000000 00z0001000
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00??0000?? ??????000? 0000000000 0010000?z0 010???????

Piabina argentea

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0101100001 0010000010 1010001011 0100110000 0000000000
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0010000001 0111000010 0000001000 0010010000 010???????

Acrobrycon tarijae

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0011110011 1?????000? 1000000001 1010000102 010???????

Knodus tanaothoros

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1000010110 000???0001 1200000001 10?01????0 0??0000000
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Piabarchus analis
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0010100000 0000110000 0100000000 z001111110 0000001010
10z001z101 000000?001 1200000001 10?011???0 0??0000000
?00000000? ??????000? 100000000z 1010001000 010???????

Hemibrycon surinamensis
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z0??100010 0000110000 0100000000 1000011110 0000001010
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Bryconamericus lethostigmus
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0010100010 0000110000 0100000000 1000011z00 0000001010
100001z111 0001zz0001 1200000001 100011??00 0100000000
0010000001 0?????000? ?000000000 1010000000 010???????

Pseudocorynopoma doriae
?00110010? 0001101010 0z00110000 1000010000 0000z000?0
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000110z001 001z000010 101000101z 0100??0010 0000000110
0010?00000 0000110010 1100000000 110011111z 0000101010
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Bryconamericus pectinatus
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0001100001 0010000011 1010001010 0100100000 0000110000

1000100000 0000110000 0100000000 1000011000 1000001010
10z00000?? ???????001 1200000001 110?1?1?00 0??0000000
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Creagrutus affinis
????????0? ???1?????? ?00??11??? ?????????? ??????????
?????????? ?????????? 1??????0?? ??????????1 00????????
??????0??? ???0????10 0011?0???0 0???11???? ???1?0????
?????0???0 0?1??0??1? ?????????? ?????0???? ??????????
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?????????? ?????????? ??????000? ?????????? ???000000?
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Bryconamericus indefessus
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Bryconamericus exodon
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Creagrutus amoenus
????????0? ???0?????? ?00??11??? ?????????? ??????????
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Creagrutus atratus
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Creagrutus barrigai

????????0? ???1?????? ?00??11??? ?????????? ??????????
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Creagrutus beni

????????0? ???0?????? ?00??11??? ?????????? ??????????
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Creagrutus bolivari

????????11 ???1?????? ?00??11??? ?????????? ??????????
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?????0????0 0?1??0??1? ?????????? ?????1???? ?????1z???
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Creagrutus brevipinnis

????????0? ???1?????? ?00??11??? ?????????? ??????????
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Creagrutus britskii

????????11 ???1?????? ?00??11??? ?????????? ??????????
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Creagrutus calai

????????0? ???1?????? ?00??11??? ?????????? ??????????
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Creagrutus caucanus
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Creagrutus changae
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Creagrutus cochui
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Creagrutus crenatus
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Creagrutus ephippiatus
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Creagrutus figueiredoi
?????????11 ???0?????? ?00??11??? ?????????? ??????????
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Creagrutus flavescens
?????????0? ???1?????? ?00??11??? ?????????? ??????????
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???????111 000?z0???? ??????000? ?????????? ???0000000?
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Creagrutus gracilis
?????????11 ???1?????? ?00??11??? ?????????? ??????????
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?????0???0 0?1??0??1? ?????????? ?????1???? ?????10???
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???????0?? ?????????? ??????000? ?????????? ???0000000?
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Creagrutus gyrospilus
?????????0? ???1?????? ?00??11??? ?????????? ??????????
?????????? ?????????? 1??????1?? ??????????1 00????????
??????0??? ???0????10 0011?0????0 0???11z010 ????1?0????
?????1???0 0?1??0??1? ?????????? ?????0???? ?????1z???
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Creagrutus hildebrandi
?????????0? ???1?????? ?00??11??? ?????????? ??????????
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?????0???0 0?1??0??1? ?????????? ?????0???? ??????????
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?????????? ???????0?? 01???0???? ????0????? ??????1???

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??????????    ??????????    ??????000?    ??????????    ???0000000?
?0???0?0???    ???????011    0111011110    0101100100    010???????
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Creagrutus holmi

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?????????0?    ???1??????    ?00??11???    ??????????    ??????????
??????????    ??????????    1??????1??    ??????????1    00????????
??????0???    ???0????10    0011?0????0    0???11z010    ???1?0????
?????0????0    0?1??0??1?    ??????????    ?????0????    ?????1z???
????0?0???    ??????????    ???????0??    ??????????    ????01?0??
????????z0    ?????000??    01???0????    ????00z000    ??????1???
???????111    000?z0????    ??????000?    ??????????    ???0000000?
?0???0?0???    ???????011    0111011110    0101100100    010???????
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Creagrutus hysiginus

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?????????0?    ???0??????    ?00??11???    ??????????    ??????????
??????????    ??????????    1??????1??    ??????????1    00????????
??????0???    ???0????10    0011?0????0    0???111010    ???1?0????
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Creagrutus ignotus

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?????????0?    ???1??????    ?00??11??0    ??????0???    ??????????
??????????    ??????????    1??????1??    ??????????1    00????????
??????0???    ???0?0??10    0011?0????0    0???1100?0    ???1?0????
??1??0????0    0?1??0??1?    ??????????    ?????0????    ?????1z0??
????0?1???    ??????????    ???????0??    ??????????    ????01?0??
????????10    ??00?000??    01???0????    ????00z000    ??????1???
??????????    ???????0??    ??????000?    ??????????    ???0000000?
?0?0000???    ???????011    0111011110    0101100100    010???????
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Creagrutus kunturus

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?????????0?    ???1??????    ?00??11???    ??????????    ??????????
??????????    ??????????    1??????1??    ??????????1    00????????
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?0???0?0???    ???????011    0111111110    0101100100    110???????
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Creagrutus lassoi

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?????????0?    ???0??????    ?00??11???    ??????????    ??????????
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Creagrutus lepidus

????????0?	???0??????	?1???11???	??????????	??????????
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Creagrutus machadoi

????????0?	???1??????	?00??11???	??????????	??????????
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Creagrutus magdalenae

????????0?	???1??????	?00??11???	??????????	??????????
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Creagrutus magoi

????????0?	???1??????	?00??11???	??????????	??????????
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?0???0?0???	???????011	0111011110	0101100100	011????????

Creagrutus manu

????????0?	???1??????	?00??11???	??????????	??????????
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Creagrutus maxillaris

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Creagrutus melanzonus

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?????????0?   ???1??????    ?00??11???    ??????????    ??????????
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Creagrutus melasma

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Creagrutus menezesi

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Creagrutus mucipu

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Creagrutus nigrostigmatus

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Creagrutus occidaneus
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Creagrutus ortegai
?????????0? ???1?????? ?00??11??? ?????????? ??????????
?????????? ?????????? 1??????1?? ??????????1 00????????
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Creagrutus paraguayensis
????????11 ???1?????? ?00??11??? ?????????? ??????????
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Creagrutus paralacus
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Creagrutus pearsoni
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Creagrutus petilus

????????0?	???0??????	?00??11???	??????????	??????????
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Creagrutus phasma

????????0?	???1??????	?00??11???	??????????	??????????
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Creagrutus pila

????????0?	???0??????	?10??11???	??????????	??????????
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Creagrutus provenzanoi

????????0?	???0??????	?00??11???	??????????	??????????
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Creagrutus runa

????????0?	???1??????	?00??11???	??????????	??????????
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?0???0?0???	???????010	0111011110	0101100100	010????????

Creagrutus saxatilis

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????????0?    ???0??????    ?00??11???    ??????????    ??????????
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Creagrutus seductus
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Creagrutus ungulus
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Creagrutus vexillapinnus
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??????0???    ???0????10    0011?0???0    0???110010    ???1?0????
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Creagrutus zephyrus
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Creagrutus guanes
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?????0???0 0?1??0??1? ?????????? ?????1???? ?????1z???
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Diapoma alburnus
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0010100000 0000110000 0100000000 1000011100 0000001010
1000010111 0000000001 1200000001 0000111000 0100000000
0010000001 0?????000? 1000000001 1010001000 0100z00000

Diapoma itaimbe
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0011000000 0010100101 1?00000000 00011??011 0010100001
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Diapoma dicropotamicus
?00110010? 0001101010 0000110000 1100010000 0000101100
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0001100001 0010000011 1010001010 0100100000 0000100000
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Diapoma alegretensis
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Diapoma uruguayensis
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Diapoma lepiclastus
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0001100001 0010000011 1010001010 0100??0000 000z100100
0000100000 0000110000 0100000000 1001111110 0000001010
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Diapoma tipiaia
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1000010??? ???????001 1200000000 ?????????0 0100000000
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Diapoma sp. Iguacu
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0000100000 0000110000 0100000000 1001011100 0000001010
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Diapoma obi
?00110010? 0001101010 0000110000 1100010000 0000101z00
0011000000 0010000z0z 1?00000000 00011??011 010?100001
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zz10100000 0000110000 0100000000 z0010111z0 0000001010
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1000000?? ??????000? 1000000001 101000z10? 0101101010

Diapoma thauma
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Diapoma pyrrhopteryx

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Diapoma terofali

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0011000000 0010100001 1?00000000 00011??011 010?100001
0001000001 z100000010 001000110z 000011z0z0 0001?00000
0101000000 0010000010 100z100011 0010100010 1000110000
0001100001 0010000010 1010001010 0100??0000 0000100100
0110100000 0000110000 0100000000 1z01111110 0000001010
1000010111 0001100001 1200000?1 0000111000 0100000000
0001110011 1?????000? 1000000001 1010001000 0101100000

Diapoma speculiferum

?00110010? 0001101010 0000110000 1100010000 0000101100
0011000000 0010000001 1?00000000 00011??011 010?100001
0001000001 1100000010 001000110z 0000111010 0001?00000
0101000000 0010000010 1000100011 001z1000z0 1z00110000
0001100001 001000001z 1010001010 0100??0000 0000100100
0000100000 0000110000 0100000000 1z01111110 0000001010
1000010111 0000100001 1200000?1 0000111000 0100000000
0011110011 1?????000? 1000000001 1010001000 0101110000

Diapoma guarani

?00110010? z001101010 0z0?110000 1100010000 0000101100
0011000000 0010100z0z 1?00000000 00011??011 010?100001
0001000001 1100000010 0010001100 0000111010 0001?00010
0101000000 0010000010 101z100011 0011100000 1001110000
0001100001 0010000011 1010001010 0100??0000 0000100100
00z0100000 0000110000 0100000000 10011111z0 0000001010
1000010111 0000000001 0200000001 0000111000 0100000000
1000000?11 1?????000? 1000000001 0010001000 0101z00011

Diapoma sp.

?00110010? z001101010 0z0?110000 1100010000 0000101100
0011000000 0010100001 1?00000000 00011??011 010?100001
0001000001 1100000010 0010001100 00001100z0 0001?00010
0101000000 0010000010 1000100011 00111000z0 1001110000
0001100001 001z000011 1010001010 0100??0000 0000100100
00z0100000 0000110000 0100000000 1z011111z0 0000001010
1000010111 0000000001 0200000001 0000111000 0100000000
1010000?10 0?????000? 1000000001 0010001000 0101z00010

Appendix S3 Complete list and data matrix of continuous morphological characters.

1. Number of scales sheath on anal-fin base.

2. Number of anal-fin branched rays.

3. Number of scale rows between lateral line and dorsal-fin origin.

4. Percentage of the distance of snout to pelvic-fin origin in the SL.

5. Anal-fin base length in the SL.

6. Body height in the SL.

7. Bony head length in the SL.

8. Upper jaw length in the head length.

9. Number of cusps teeth of outer series of premaxilla.

10. Number of cusps teeth of inner series of premaxilla.

11. Number of maxillary teeth.

12. Number of cusps teeth of maxilla.

13. Number of dentary teeth.

14. Number of cusps teeth of dentary.

Species/Characteres	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Argopleura magdalenensis	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Attonitus ephimeros	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Aulixidens eugeniae	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Bryconamericus agna	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Knodus alpha	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Bryconamericus cf. iheringii	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Bryconamericus cf. rubropictus	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Bryconamericus mennii	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Bryconamericus rubropictus	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Eretmobrycon scleroparius	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Piabina thomasi	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Hemibrycon dariensis	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Knodus breviceps	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Knodus heterestes	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Knodus meridae	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Mimagoniates rheocharis	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Eretmobrycon emperador	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Creagrutus anary	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Creagrutus atrisignum	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Creagrutus meridionalis	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Creagrutus taphorni	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Creagrutus cracentis	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Creagrutus gephyrus	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Creagrutus maracaiboensis	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Creagrutus muelleri	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Creagrutus ouranonastes	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Creagrutus peruanus	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Carlastyanax aurocaudatus	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Microgenys minuta	?	?	?	?	?	?	?	?	?	?	?	?	?	?

Piabina argentea	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Acrobrycon tarijae	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Knodus tanaothoros	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Piabarchus analis	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Hemibrycon surinamensis	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Bryconamericus lethostigmus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Pseudocorynopoma doriae	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Bryconamericus pectinatus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus affinis	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Bryconamericus indefessus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Bryconamericus exodon	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus amoenus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus atratus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus barrigai	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus beni	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus bolivari	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus brevipinnis	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus britskii	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus calai	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus caucanus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus changae	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus cochui	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus crenatus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus ephippiatus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus figueiredoi	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus flavescens	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus gracilis	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus gyrospilus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus hildebrandi	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus holmi	?	?	?	?	?	?	?	?	?	?	?	?	?	?	

Creagrutus hysiginus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus ignotus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus kunturus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus lassoi	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus lepidus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus machadoi	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus magdalenae	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus magoi	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus manu	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus maxillaris	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus melanzonus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus melasma	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus menezesi	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus mucipu	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus nigrostigmatus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus occidaneus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus ortegai	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus paraguayensis	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus paralacus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus pearsoni	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus petilus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus phasma	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus pila	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus provenzanoi	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus runa	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus saxatilis	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus seductus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus ungulus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus vexillapinnus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
Creagrutus zephyrus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	

Creagrutus guanes	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Diapoma alburnus	6-8	20-23	5-6	?	?	?	?	?	?	3-5	3-7	1-3	8-13	3-5
Diapoma itaimbe	5-16	21-27	5-7	42.5-48.5	28.3-36.1	26.0-34.5	20.0-25.3	31.3-45.9	3	3-5	5-7	1-3	13-17	3-5
Diapoma dicropotamicus	9-12	22–26	5-6	41.6-45.6	28.8-34.1	26.6-33.1	22.1-24.8	33.3-41.9	3	3-5	4-6	1-3	10-14	3-5
Diapoma alegretensis	12-18	23-30	5-6	38.9-43.5	32.6-38.8	29.8-36.4	21.5-24.0	36.9-44.6	3	5	4-6	1-5	10-14	5
Diapoma uruguayensis	20-30	29-35	5-7	38.5-45.3	33.2-43.1	29.4-39.3	20.7-25.9	31.9-43.2	3	5-7	4-7	3-5	12-14	5-7
Diapoma lepiclastus	13-20	24-29	6–7	38.6-44.6	32.8-40.0	27.6-37.7	20.9-24.5	34.1-44.2	3	3-5	3-8	3	11-14	3-5
Diapoma tipiaia	7-10	21-23	4-5	42.3-45.3	27.9-31.3	28.5-31.3	21.5-23.4	40.5-48.2	3	3	2-3	1-3	10-11	3
Diapoma sp. Iguaçu	5-9	22-26	?	?	?	?	?	?	3	3-5	3-4	1-3	7-10	4-5
Diapoma obi	7-14	22-24	5-6	44.4-50.6	29.2-34.1	34.5-40.8	21.3-24.3	42.0-46.9	3	3-5	3-6	1-4	12	3-5
Diapoma thauma	?	24-27	?	44.4–50.0	23.8-34.8	23.8-31.1	23.2-28.5	37.3-43.8	3	3-4	2-5	3	9-15	3-4
Diapoma pyrrhopteryx	?	24-30	?	44.8-50.5	30.3-34.6	25.1-32.5	24.5-27.2	41.0-46.4	3	5	3-6	4-5	8-14	4-5
Diapoma terofali	?	26-33	?	46.7-51.0	30.8-36.6	29.3-37.5	22.4-26.0	43.2-48.4	?	?	1-6	1-3	8-15	4-5
Diapoma speculiferum	?	25-32	?	44.1–49.6	30.3-35.8	25.2-32.8	23.6-27.3	38.8-44.8	3	5	2-8	3	8-15	4-5
Diapoma guarani	7-13	19-25	5-6	46.9-52.7	27.9-35.3	29.2-35.9	21.525.4	34.4-42.9	3	3	2-4	1-3	10-14	3
Diapoma sp.	7-10	20-25	5-6	44.1-51.9	29.4-33.3	29.9-33.3	21.8-24.9	29.0-39.1	1	3	2	1	8-10	3

Appendix S4 Additional table (S1)

Table S1 Primers used in this study, with their sequence, if 1st or 2nd PCR, references and PCR conditions.

Gene	Primer sequence (liste from 5' to 3')	PCR	Reference	PCR Conditions
COI_FishF2_t1	CAGGAAACAGCTATGACACTTCAGGGTG			
COI_ VF2_t	CAGGAAACAGCTATGACACCTCAGGGTG	1st	Ward et al. (2005)	40x (94°C/30", 50°C/20", 48°C/5", 46°C/5",
COI_FishR2_t1	TGTAAAACGACGGCCAGTGACTAATCA			44°C/5", 42°C/5", 40°C/20", 72°C/1') 72°C/3'
COI_FR1d_t1	TGTAAAAGGACGGCCAGTCAACCAACCA		Ivanova et al. (2007)	
16Sar_L	ACG CCT GTT TAT CAA AAA CAT	1st	Palumbi (1996)	30x (95C-30", 55C-30", 72C-1') 72C-5'
16Sbr_H	CCG GTC TGA ACT CAG ATC ACG T			
MYH6_F459	CATMTTYTCCATCTCAGATAATGC	1st		30x (94C/30", 53C/45", 72C/1'30") 72C/5'
MYH6_R1325	ATTCTCACCACCTCCAGTTGAA			
MYH6_F507	GGAGAATCARTCKGTGCTCATCA	2nd	Li *et al*. 2007	32x (94C/32", 60/45", 72C/1'32") 72C/5'
MYH6_R1322	CTCACCACCATCCAGTTGAACAT			
SH3PX3_F461	GTATGGTSGGCAGGAACYTGAA	1st		30x (94°C/30", 55°C/45", 72°C/1'30") 72°C/5'
SH3PX3_R1303	CAAACAKCTCYCCGATGTTCTC			
SH3PX3_F532	GACGTTCCCATGATGGCWAAAAT	2nd		30x (94°C/30", 65°C/45", 72°C/1'30") 72°C/5'
SH3PX3_R1299	CATCTCYCCGATGTTCTCGTA			

References

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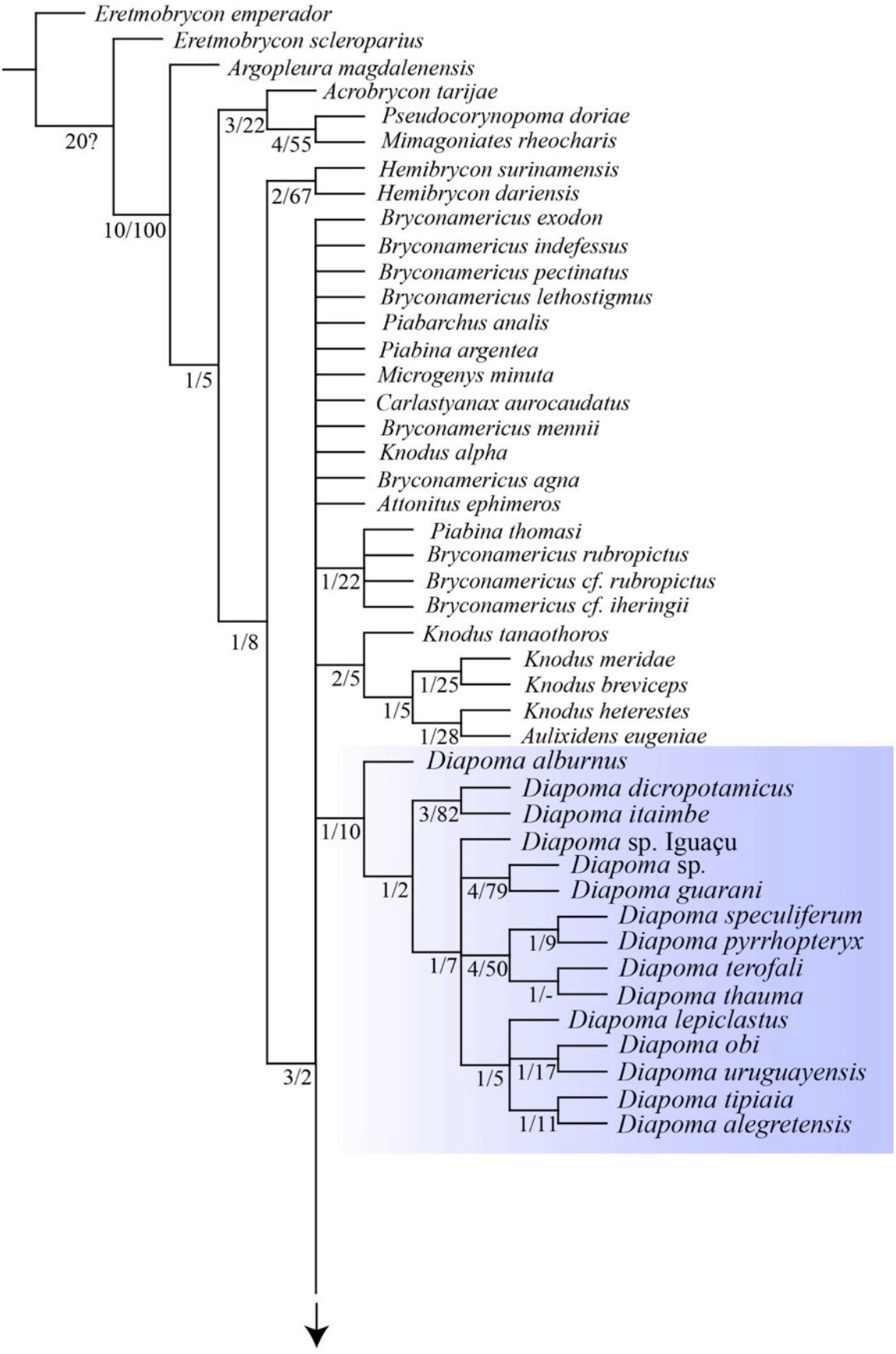
Ward, R.D., T. S. Zemlak, B. H. Innes, P. R. Last & P. D. N. Hebert. 2005. DNA barcoding Australia's fish species. Philosophical Transactions of The Royal Society B, 360: 1847-1857.

Appendix S5 Additional figures (S1 and S2).



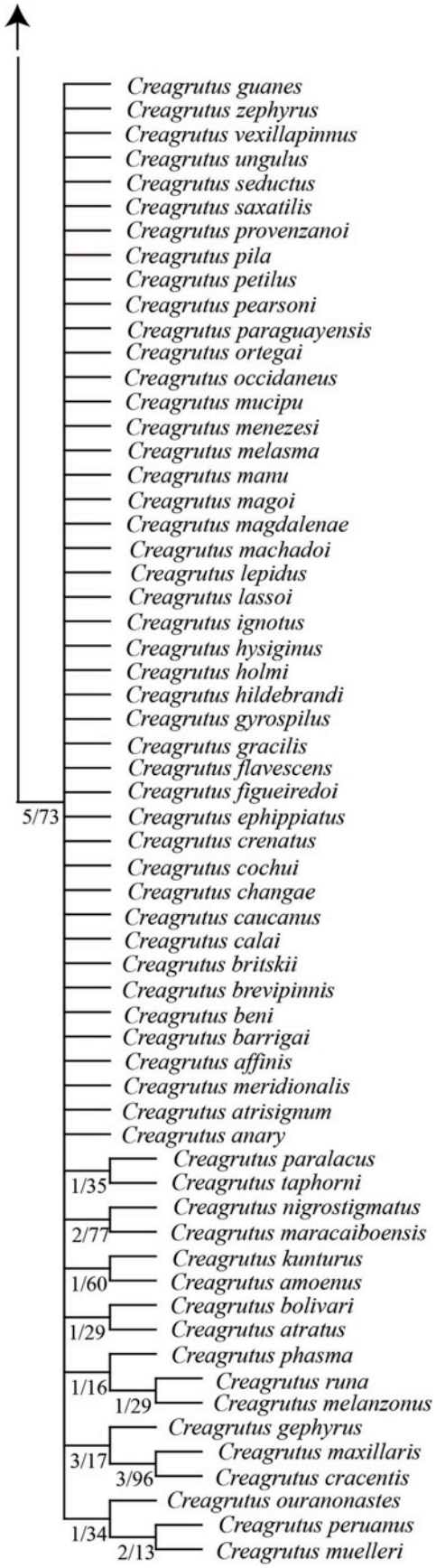


Fig. S1 Cladogram generated through parsimony with discrete morphological characters showing relationships of the *Diapoma* species with remaining Stevardiinae. Node numbers correspond to Bremer support and Bootstrap value.

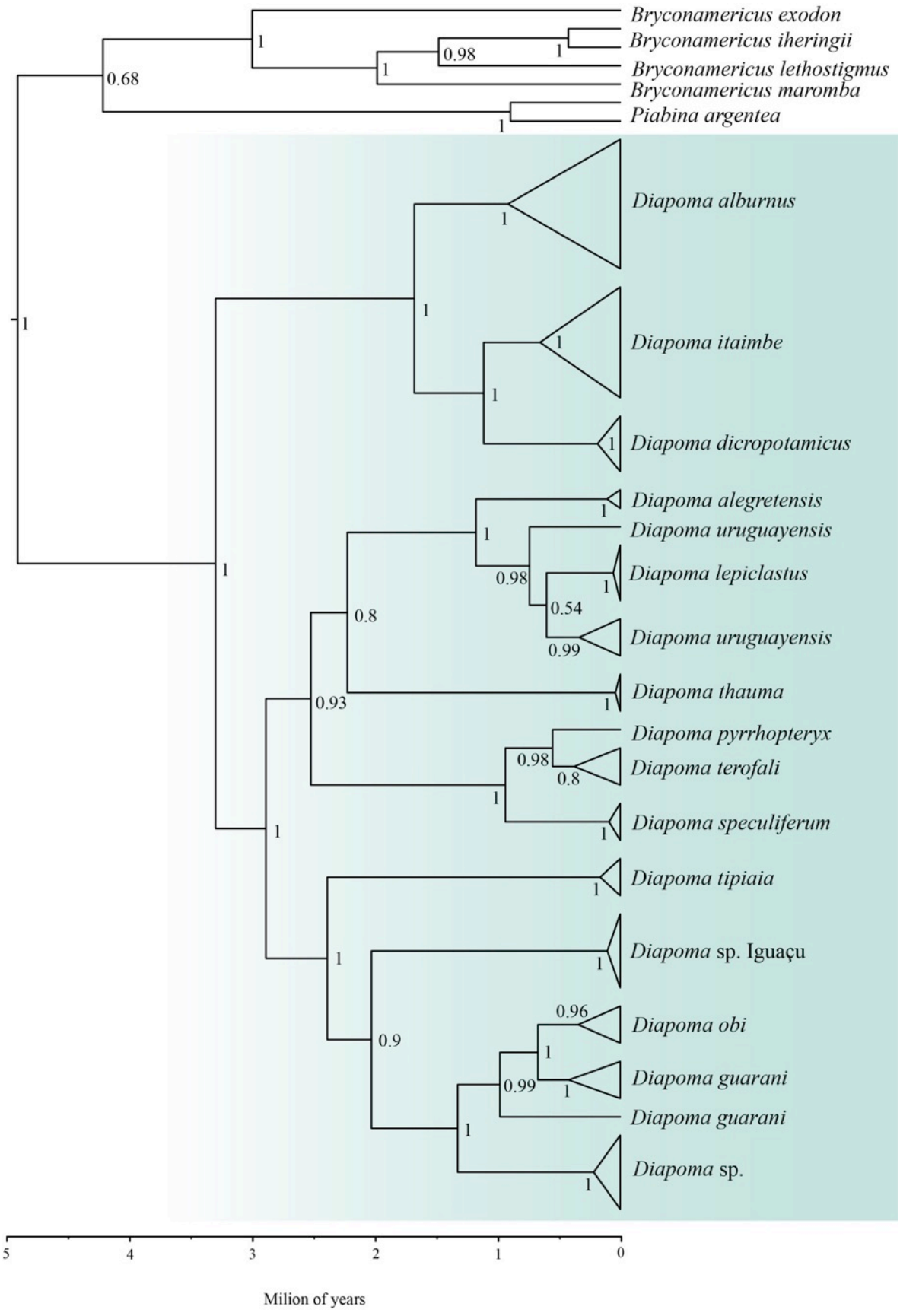


Fig. S2 Cladogram generated through bayesian inference with mtDNA (*COI* and *16S*) and nDNA (*Myh6* and *SH3PX3*) showing relationships of the *Diapoma* species. Node numbers correspond to posterior probabilities.

Capítulo Conclusivo

CONCLUSÃO

A tese ora apresentada analisou a história evolutiva e relações entre as populações de duas espécies simpátricas, *Diapoma itaimbe* e *Bryconamericus lethostigmus*, e as relações filogenéticas das espécies de *Diapoma*.

As análises moleculares de *Diapoma itaimbe* indicaram três grupos evolutivos que divergiram entre aproximadamente 1,6 e 0,45 Ma. A população com divergência mais antiga (Rio Araranguá) apresenta tempo estimado de divergência similar ao encontrado entre outras duas espécies do mesmo gênero (*D. alburnus* e *D. dicropotamicus*). Porém, apesar de uma forte estruturação genética, a morfologia geral da espécie é conservada nas populações. Assim, de acordo com os resultados, consideramos *D. itaimbe* como uma espécie com populações estruturadas na qual uma delas (Rio Araranguá) pode estar em processo de especiação devido ao isolamento geográfico entre as populações. A estruturação populacional encontrada em *D. itaimbe* pode ser explicada por barreiras ecológicas que impedem o fluxo gênico entre as drenagens da ecorregião Tramandaí-Mampituba, mesmo entre aquelas que apresentam conexão atual através das lagoas costeiras, como é o caso das populações do Rio Maquiné e Rio Três Forquilhas. Isto também indica que, no passado, as ligações fluviais na planície costeira devido aos recuos do nível do mar podem ter sido seletivas e, assim, podem não ter representado corredores de dispersão para toda a ictiofauna. Entretanto o compartilhamento de haplótipos entre as populações do Rio Três Forquilhas e Rio Mampituba, apesar destas populações estarem em diferentes drenagens, pode indicar uma relação ancestral entre elas ou fluxo gênico recente, devido à captura de cabeceiras, um processo que ainda está ativo nos rios da região costeira.

Considerando que *B. lethostigmus* e *D. itaimbe* ocorrem nas mesmas drenagens, são sintópicas e compartilham uma história de vida semelhante, a diferença nos padrões filogeográficos encontrados para ambas as espécies é notável. Enquanto *D. itaimbe* mostra uma forte estrutura genética com clados mtDNA bem definidos, *B. lethostigmus* mostrou ausência de estrutura genealógica e apenas uma estrutura genética muito fraca com base nos dados mtitocondriais. Tal diferença entre estas espécies podem ser explicadas por três diferentes cenários, ou por uma combinação deles: (1) diferentes taxas evolutivas do mtDNA entre as duas espécies; (2) tempos da colonização diferentes das duas espécies nestas drenagens; e (3) fluxo gênico entre populações de *B. lethostigmus* através paleodrenagens dificultando a estruturação das populações. No entanto, de acordo com os resultados gerados, o cenário 2 parece ser o mais provável. *Bryconamericus lethostigmus* e *D. itaimbe* são

espécies relacionadas e com similar tempo de geração o que torna pouco provável uma grande diferença na taxa evolutiva do mtDNA entre elas (cenário 1). Além disso, a manutenção do fluxo gênico entre populações de *B. lethostigmus* através dos paleocanais (cenário 3) geraria um grande número de haplótipos compartilhados entre as drenagens, o que não foi observado. Este estudo analisou ainda aspectos morfológicos de *B. lethostigmus* corroborando a hipótese de que a única série de dentes no premaxilar da espécie é originada pela fusão da série externa com a série interna durante o desenvolvimento ontogenético. Assim, as observações aqui apresentadas não corroboram a hipótese primária de homologia entre as modificações bucais encontradas em *B. lethostigmus* e o gênero *Monotocheirodon*. Por fim, comparações morfológicas e moleculares entre drenagens de ocorrência de *B. lethostigmus* refutam a hipótese de que as populações do Rio Araranguá corresponderiam a uma nova espécie.

Concluindo, um outro capítulo da tese se dedicou a testar o monofiletismo de *Diapoma* (incluindo *Cyanocharax*), que foi corroborado através de análises independentes, bem como por análise de evidência total, considerando simultaneamente dados moleculares e morfológicos. No entanto, as relações entre as espécies de *Diapoma* geradas por análises morfológicas e moleculares independentes mostraram diferentes arranjos entre as espécies, sendo que as filogenias moleculares apresentaram maior suporte para os clados do que as filogenias morfológicas. A limitada variação morfológica, além de processos de convergência entre as espécies observadas neste estudo, resultaram em um alto número de homoplasias e consequente diminuição da resolução filogenética, revelando assim a necessidade de buscar novos caracteres através de uma investigação morfológica mais exaustiva. No entanto, a filogenia gerada por evidência total apresentou uma boa resolução para as relações internas das espécies de *Diapoma*, onde foi possível inclusive traçar as sinapomorfias morfológicas características de cada clado.