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REVISÃO TAXONÔMICA E FILOGENIA DE CHEIRODONTINAE (CHARACIFORMES: CHARACIDAE): INTEGRANDO EVIDÊNCIA MORFOLÓGICA E MOLECULAR

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 Doutor em Biologia Animal.

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Orientador: Prof. Dr. Luiz Roberto Malabarba

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RESUMO

A ictiofauna neotropical de água doce é taxonomicamente a mais diversa do planeta, porém sua diversidade ainda é amplamente subestimada. A evidência para essa subestimativa vem do acúmulo de formas morfologicamente distintas, mas não descritas, depositadas em coleções de museus e dos resultados de estudos baseados em DNA (por exemplo, delimitação de espécies) que identificam consistentemente um número maior de linhagens divergentes do que o atualmente é aceito, mesmo dentro de grupos bem estudados. Investigamos a diversidade de linhagens dentro de Cheirodontinae, sequenciamos a subunidade I do citocromo c oxidase mitocondrial (COI) e delineamos linhagens usando 8 diferentes métodos de delimitação de espécies de lócus único. Os resultados fornecem evidências fortes e consistentes para a existência de uma diversidade adicional e não descrita dentro de Cheirodontinae. Dentro de Cheirodontinae, encontramos hipóteses divergentes (morfológicas vs. moleculares) sobre as relações filogenéticas dentro da subfamília. Testamos essas duas hipóteses, usando dados integrados (morfológicos e moleculares: COI, 16S, 12S, RAG1, RAG2, Myh6) propomos uma nova hipótese de relações filogenéticas dentro da subfamília, e subdividimos a subfamília, em 8 clados, que são corroborados pelas diferentes metodologias (parcimônia e modelos). Com esta informação morfológica e molecular, estimamos o tempo de divergência dentro da subfamília usando Evidencia-total com datação FBD, o que nos levou a determinar que Cheirodontinae se diversificou de Aphyocharacinae, aproximadamente 28 Ma. Com esses resultados reconstruímos a história biogeográfica dos Cheirodontinae na região neotropical e testamos a influência do tamanho e sua capacidade de inseminação em sua diversificação.

Palavras chave: Biogeografia, evolução, sistemática, neotropical, peixes

ABSTRACT

The neotropical freshwater fish fauna is taxonomically the most diverse vertebrate on the planet, however its diversity is still largely underestimated. Evidence for this underestimation comes from the accumulation of morphologically distinct but undescribed forms deposited in museum collections, and from the results of various DNA-based studies (eg, species delimitation) that consistently identify a larger number of divergent lineages than currently accepted, even within well-studied species, we investigated the diversity of lineages within the Cheirodontinae. To investigate this diversity, we sequenced the mitochondrial cytochrome c oxidase subunit I (COI) and delineated lineages using 8 different single-locus species delimitation methods. The results provide strong and consistent evidence for additional and undescribed taxonomic diversity in Cheirodontinae. Within the Cheirodontinae, we find divergent hypotheses (morphological vs. molecular) about phylogenetic relationships within the subfamily. We test these two hypotheses, using integrated data (morphological and molecular: COI, 16S, 12S, RAG1, RAG2, Myh6) we propose a new hypothesis of phylogenetic relationships within the subfamily, and we subdivide the subfamily, into 8 clades, which are corroborated by the different methodologies (parsimony and models). With this morphological and molecular information, we estimated the time of divergence within the subfamily using Total-evidence with FBD dating, this led us to determine that the Cheirodontinae diversified from the Aphyocharacinae, approximately 28 Ma. With these results we reconstruct the biogeographical history of the Cheirodontinae in the neotropical region and we test the influence of the size and its insemination capacity in its diversification.

Keywords: Biogeography, evolution, systematics, neotropical, fish

INTRODUÇÃO

A subfamília Cheirodontinae apresenta uma ampla distribuição nas principais drenagens da América Central e do Sul, ocorrendo desde a Costa Rica até o centro do Chile e Argentina, em ambos lados dos Andes (Malabarba, 2003). Atualmente, está composta por 66 espécies válidas em 16 gêneros: *Acinocheirodon* Malabarba & Weitzman, 1999, *Aphyocheirodon* Eigenmann, 1915, *Cheirodon* Girard, 1855, *Cheirodontops* Schultz, 1944, *Compsura* Eigenmann, 1915, *Ctenocheirodon* Malabarba & Jerep, 2012, *Heterocheirodon* Malabarba, 1998, *Kolpotocheirodon* Malabarba & Weitzman, 2000, *Macropsobrycon* Eigenmann, 1915, *Nanocheirodon* Malabarba, 1998, *Odontostilbe* Cope, 1870, *Prodontocharax* Eigenmann & Pearson, 1924, *Protocheirodon* Vari, 2016, *Pseudocheirodon* Meek & Hildebrand, 1916, *Saccoderma* Schultz, 1944, *Serrapinnus* Malabarba, 1998, e uma espécie fóssil no gênero *Megacheirodon* MC Malabarba. 1998.

Ao longo de sua história diversos autores tentaram definir Cheirodontinae, com métodos e esquemas de classificação diferentes. Eigenmann (1915) agrupa de forma artificial esta subfamília em 21 gêneros incluindo 56 espécies: *Aphyocharax* Günther, 1868, *Aphyocheirodon*, *Aphyodite* Eigenmann, 1912, *Cheirodon*, *Compsura*, *Grundulus* Valenciennes, 1846, *Holoshesthes* Eigenmann, 1903, *Leptagoniates* Boulenger, 1887, *Leptobrycon* Eigenmann, 1915, *Macropsobrycon*, *Megalamphodus* Eigenmann, 1915, *Microschemobrycon* Eigenmann, 1915, *Mixobrycon* Eigenmann, 1915, *Odontostilbe*, *Oligobrycon* Eigenmann, 1915, *Paragoniates* Steindachner, 1876, *Parecbasis* Eigenmann, 1914, *Phenagoniates* Eigenmann & Wilson, 1914, *Prionobrama* Fowler, 1913, *Probolodus* Eigenmann, 1911 and *Spintherobolus* Eigenmann, 1911, gêneros que inicialmente foram classificados como Aphyocharacinae (Eigenmann, 1909). Posteriormente, muitos pesquisadores começaram a descrever espécies novas, que foram adicionadas à subfamília Cheirodontinae por apresentar uma fileira de dentes na pré-

maxila: *Pseudocheirodon* ; *Monotocheirodon* Eigenmann & Pearson, 1924; *Prodontocharax*, *Rachoviscus* Myers, 1926, *Atopomesus* Myers , 1927, *Othonocheirodus* Myers, 1927), *Amblystilbe* Fowler, 1940, *Pedalibrycon* Fowler, 1943, *Saccoderma*, *Cheirodontops*, *Odontostoechus* Gomes, 1947, *Distoechus* Gomes, 1947, *Aulixidens* Böhlke, 1952, e *Thrissobrycon* Böhlke, 1953. Fowler (1958) considera válido Cheirodontidae, dividindo-o em três tribos Glandulidi, Aphyocharacidi e Cheirodonidi. Géry (1960) discute a situação de Cheirodontinae, com o incremento de novas espécies dentro da subfamília, apontando que é difícil definir os limites de Cheirodontinae definidos por Eigenmann (1915) e que Cheirodontinae poderia ser reunido em uma única subfamília, Tetragonopterinae, dividida em três grupos: Cheirodontidi (incluindo *Cheirodon*, *Odontostilbe*, *Aphyocheirodon*, *Holoshesthe*s, *Cheirodontops*, *Aulixidens*, *Othonocheirodus*, *Odontostoechus* e *Monotocheirodon*), Aphyocharacidi (incluindo *Aphyocharax*, *Grundulus*, *Spintherobolus*, *Phoxinopsis*, *Probolodus Atopomesus*, *Prodontocharax*, *Parecbasis*, *Thrissobrycon*, etc), e um terceiro grupo que inclui os "Gênero de borda (*Pristella*, *Megalamphodus*, *Aphyocharacidium* e *Pseudopristella*).

Fink & Weitzman (1974) revisaram as espécies de Cheirodontinae da América Central considerando o grupo polifilético. Os mesmos autores sinonimizaram os gêneros *Odontostilbe*, *Pseudocheirodon* e *Compsura* em *Cheirodon*, baseado na reavaliação de caracteres. Géry (1977) menciona que para aceitar a hipótese de Fink & Weitzman (1974) é necessária uma revisão completa do gênero incluindo as espécies tipo (*Cheirodon pisciculus* e *Odontostilbe fugitiva*) e considera válido o gênero *Odontostilbe*.

Weitzman & Fink (1983) discutem a classificação de Eigenmann (1915, 1917) por classificar o gênero de Characiformes seguindo dois critérios: um grupo de espécies estreitamente relacionadas descendentes de um antepassado comum e outro de tipo polifilético, consistem em espécies que tem una combinação de caracteres em comum.

Este último critério de classificação causou muitos problemas para o reconhecimento do género nos anos subsequentes, como foi o caso dentro de Cheirodontinae. Além de mencionar que para reconhecer como válido *Odontostilbe* e *Cheirodon* é necessário realizar uma revisão taxonômica de Cheirodon e estudar as relações filogenéticas de todas as espécies atualmente alocadas no gênero.

Uj (1987) revisou as espécies de Cheirodontinae do Paraguai redefinindo *Odontostilbe* e *Cheirodon*, baseado na análise das espécies tipo (*Cheirodon pisciculus* e *Odontostilbe fugitiva*) e agrupou espécies dentro destes gêneros por similaridade morfológica, como por exemplo: *Cheirodon piaba*, *C. kriegi*, *C. stenodon*, *C. microdon* e *C. notomelas* transferidas para *Odontostilbe*.

Malabarba (1998) propõe quatro sinapomorfias que suportam a monofilia de Cheirodontinae: presença de um hiato grande, quase triangular, dos músculos que cobrem a câmara anterior da bexiga natatória entre a primeira e a segunda costela pleural (pseudotímpano); ausência de macula umeral; dentes pedunculados, amplamente expandidos e comprimidos distalmente; uma única fileira de dentes regulares com dentes perfeitamente alinhados e similares em forma e número de suas cúspides no pré-maxilar. Malabarba (1998) subdivide Cheirodontinae em três grupos, dois grandes grupos monofiléticos formando as tribos Cheirodontini e Compsurini e um grupo formado pelos gêneros restantes, considerados incertae sedis. Com a revisão de Malabarba (1998) e trabalhos posteriores, foram descritos sete gêneros: *Heterocheirodon*, *Nanocheirodon*, *Serrapinnus*, *Acinocheirodon*, *Kolpotocheirodon*, *Ctenocheirodon* e *Protocheirodon.*

Calcagnotto et al. (2005) corroborou a monofila de Cheirodontinae utilizando dois genes mitocondriais [16S e citocromo b (Cytb)] e quatro genes nucleares (RAG2, sia, fkh e trop) incluindo em sua análise espécies dos gêneros *Aphyocheirodon, Cheirodon*, *Cheirodontops* e *Prodontocharax*. Mirande (2009, 2010) corroborou a monofilia de

Cheirodontinae fazendo uma análise filogenética com dados morfológicos incluindo seis espécies de gêneros (*Prodontocharax, Odontostilbe, Serrapinnus* e *Cheirodon*), onde a subfamília foi hipotetizada como grupo irmão de Aphyoditeinae. Javonillo et al. (2010) em sua análise filogenética com sequências de DNA de três genes mitocondriais (12S, 16S e COI) e um nuclear (RAG2) determinou Cheirodontinae como grupo monofilético dentro do clado B conformado pelos gêneros *Exodon, Phenacogaster, Roeboides, Galeocharax, Cynopotamus, Tetragonopterus, Aphyocharax* e a subfamília Cheirodontinae. Oliveira (2011) baseado em análise filogenética com de DNA com dois marcadores mitocondriais (16S e Cytb) e três genes nucleares (Myh6, RAG1 e RAG2) determina que Cheirodontinae como um grupo polifilético rejeitando a hipóteses de Malabarba (1998), Calcagnotto et al. (2005), Mirande (2009,2010) e Javonillo (2010), excluindo *Spintherobolus* de Cheirodontinae e posicionando-o como grupo irmão dos clados 52 (Stethaprioninae, Rhoadsiinae e gêneros previamente considerados incertae sedis em Characidae), 54 (Cheirodontinae, Aphyocharacinae, Characinae, Tetragonopterinae e gêneros previamente considerados incertae sedis em Characidae) e 55 (Stevardiinae), incluindo dentro de Cheirodontinae os gêneros *Compsura, Serrapinnus, Odontostilbe, Aphyocheirodon, Macropsobrycon, Kolpotocheirodon, Cheirodon, Heterocheirodon Prodontocharax, Saccoderma, Pseudocheirodon* e *Nanocheirodon*, não suportando a divisão de Cheirodontinae nas tribos Compsurini e Cheirodontini, destacando os Cheirodontinae transandinos (*Nanocheirodon insignis* e *Pseudocheirodon arnoldi*) como grupo irmão dos Cheirodontinae cisandinos.

Mariguela et al. (2013) realizou uma análise filogenética com dois genes mitocondriais (16S e Cytb) e 3 genes nucleares (RAG1, RAG2 e Myh6) corroborando a hipótese de Oliveira et al. (2011) e rejeitando a validade das tribos Cheirodontini, Compsurini e os gêneros *Cheirodon, Compsura, Macropsobrycon, Odontostilbe* e *Serrapinnus*, além de

propor a exclusão de *Amazonspinther* e *Spintherobolus* da subfamília Cheirodontinae. Mariguela et al. (2013) também propõe remover *Macropsobrycon xinguensis* do gênero Macropsobrycon, e incluir *Leptagoniates pi* dentro de Cheirodontinae, a exclusão de *Cheirodon stenodon* de *Cheirodon* e seu inclusão dentro de Cheirodontinae como outro gênero e propõe uma nova definição das tribos monofilético: Cheirodontini, Compsurini e Pseudocheirodontini.

Jerep & Malabarba (2014), discutiram os resultados de Mariguela et al. (2013) destacando a falta de definição de caracteres que suportem as tribos Compsurini e Cheirodontini, assim como para a nova tribo proposta Pseudocheirodontini, além de sua falta de discussão de evidências que suportam o reconhecimento das tribos Cheirodontini e Compsurini sensu Malabarba (1998), mostrando como exemplo a situação de *Serrapinnus* discutindo os caracteres que suportam a validade de este gênero, que posteriormente foi corroborado em Malabarba & Jerep (2014). Mirande (2019), com seu estudo de evidencia total, usando máxima parcimônia, corrobora a hipóteses de Mariguela et al. (2013), propondo o clado *Saccoderma*, clado *Odontostilbe*, e clado *Holoshesthes.* Melo et al. (2022), com seu analises de elementos ultraconservados (UCEs), corrobora a monofilia de Cheirodontinae, sendo grupo irmão de Aphyocharacinae, entre seus localiza *Macropsobrycon uruguayanae*, como parte o clado de *Cheirodon*, resultado que contradisse todas as hipóteses previas, sobre as relações de *Macropsobrycon.*

Tendo em vista as hipóteses contraditórias a nível molecular e morfológico sobre a composição e as relações filogenéticas de suas tribos, gêneros e espécies dentro de Cheirodontinae, realizamos um extenso estudo, utilizando diversas linhas de evidencia (Morfológico e molecular), e aplicando diversas metodologias (Parcimônia e modelo). Propomos novas hipóteses sobre as relações dentro da família, e propomos uma nova classificação a nível de tribos.

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OBJETIVOS

O presente trabalho tem como objetivos realizar uma análise integrativa de dados morfológicos e moleculares, usando metodologias de Máxima parcimônia e modelos com o intuito de (1) testar a monofilia dos gêneros Cheirodontinae, (2) testar a validade das tribos propostas com dados morfológicos e moleculares dentro da subfamília Cheirodontinae, (3) investigar as relações filogenéticas entre as espécies da subfamília Cheirodontinae, (4) descrever e diagnosticar as espécies novas da subfamília.

ESTRUTURA DA TESE

A tese é composta por uma introdução geral, quatro capítulos na forma de manuscritos que serão submetidos e um que foi ja publicado, e uma conclusão geral. O conteúdo dos capítulos é o seguinte:

Capítulo 1. Este capítulo apresenta os resultados de uma análise de delimitação de espécies, utilizando como referência o marcador COI, neste capitulo demostramos a presença de uma diversidade ainda desconhecida dentro dos Cheirodontinae. Este capitulo será submetido para o Journal Systematics and Biodiversity.

Capítulo 2. Este capítulo apresenta os resultados de uma análise de relações filogenéticas com dados morfológicos e moleculares, usando como metodologia Analises de Máxima Parcimônia no TNT. Neste capitulo sugerimos a subdivisão da subfamília em oito clados, fortemente suportados. Este capitulo será submetido para revista científica Cladistics

Capítulo 3. Este capítulo apresenta os resultados de uma análise de relações filogenéticas usando modelos, determinamos o tempo de divergência do Cheirodontinae usando a evidência total com análise FBD, que utiliza dados de sequência molecular de espécies existentes e/ou dados morfológicos de espécies fósseis e existentes (ou espécies fósseis no caso de um clado totalmente extinto) e datas de fossilização de fósseis para inferir a

filogenia datada e os parâmetros macroevolutivos. Neste capitulo reconstruímos a história biogeográfica de Cheirodontinae, e avaliamos a influência do tamanho do corpo e presença de inseminação na diversificação. Este capitulo será submetido para revista científica Molecular Phylogenetics and Evolution.

Capítulo 4. Este capítulo apresenta os resultados da descrição de uma espécie nova de Cheirodontinae usando diversas linhas de evidencia (morfologia e molecular), este trabalho foi publicado na revista científica Journal fish biology.

CHAPTER 1

Species delimitation of neotropical characins (Cheirodontinae) reveals hidden diversity through DNA Barcoding

Species delimitation of neotropical characins (Cheirodontinae) reveals hidden diversity through DNA Barcoding

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ABSTRACT

The neotropical freshwater fish fauna is taxonomically the most diverse on the planet, however its diversity is still largely underestimated. Evidence for this underestimation comes from the accumulation of morphologically distinct but undescribed forms deposited in museum collections, and from the results of various DNA-based studies (e.g., species delimitation) that consistently identify a larger number of divergent lineages than currently accepted, even within well-studied species. In the present study, we investigated the diversity of lineages within the Cheirodontinae. To achieve these objectives, we analyzed DNA sequences from 288 individuals extracted from samples deposited in tissue collections as well as from sequences obtained in GenBank and BOLD, representing 51 MOTUs, distributed in 34 Neotropical biogeographical units. We sequenced the mitochondrial cytochrome c oxidase subunit I (COI) and delineated lineages using 7 different single-locus species discovery methods (ABDG:53 lineages; ASAP:23 lineages; LocMin: 68 lineages; GYMC:53 lineages; PTP with UM tree:55 lineages, bPTP with UM tree:59 lineages, PTP with RAxML tree:49 lineages, and mPTP-15 with RAxML tree: 35 lineages). The nine morphologically distinct but undescribed species were also delimited by methods of species delimitation. The results provide strong and consistent evidence for additional and undescribed taxonomic diversity in Cheirodontinae. Although not formally described in this study, these nine MOTUs (Ctko, Obur, Odchi, Opeur, Ospp, Pger, Prodosp, Smdd, and Snotm) increase the taxonomic diversity of the Cheirodontinae by 14%. Our sampling was not exhaustive, however, and we expect additional species to be discovered mainly in under-sampled genera not included in this study such as *Saccoderma* and *Nanocheirodon*, as well as from poorly-sampled regions such as the Amazon, Orinoco, and Magdalena Basins.

Introduction

The Neotropical region hosts a high diversity of freshwater fish, with an estimated 8,000-9,000 species (Reis et al. 2016), and currently presents various types of deficiencies that limit the knowledge of the real diversity of fish in the region (Hortal et al. 2015). Throughout history, various geological processes such as the formation of the Andes, variation in sea level, effect of paleodrainages, capture of headwaters, and other climatic events have favored the diversification of fish in the region, not only in vicariance but also in the geo-dispersion of entire communities, as well as the formation of heterogeneous habitats, which influence the current diversity of fish (Ribeiro, 2006; Albert & Reis, 2011; Thomaz et al. 2015, Thomaz et al. 2017).

One of the most diverse groups within the Characidae family is the Cheirodontinae subfamily, embodying 66 valid species and 16 genera (Fricke et al. 2022). Members of the subfamily Cheirodontinae can be distinguished from all other Characiformes by the following characters: a pseudotympanum present between the first and second pleural ribs, no humeral spot, mandibular teeth with a basal peduncle with a highly compressed and generally expanded distal tip with several cusps, presence of a perfectly aligned row of teeth in the premaxilla, similar in shape and number of cusps (Malabarba, 1998).

In addition to their peculiar morphology, some genera such as *Acinocheirodon* Malabarba & Weitzman 1999, *Compsura* Eigenmann 1915, *Kolpotocheirodon* Malabarba & Weitzman 2000, *Macropsobrycon* Eigenmann 1915, and *Saccoderma* Schultz 1944 show remarkably specialized traits for insemination, characterized by the transfer of spermatozoa from the testes of mature males to the ovaries of mature females (Malabarba, 2003) and with round to moderately elongate spermatozoa (de Oliveira et al. 2008). This specialization has been discovered mainly from histological examination and

the observation of spermatozoa in the ovaries of females (Burns et al., 1997). Among inseminating characid species, *Compsura heterura* Eigenmann 1915 is the only documented case of internal fertilization, in which fertilization takes place very quickly just before the oviposition within the female reproductive system when the ovules enter the lumen of the ovary where the sperm are stored (Fukakusa et al. 2020). Specializations of the genital and anal papillae of females and males are involved in the copulation of *C. heterura*, varying throughout development and during copulation and spawning (Lezama & Malabarba, 2021).

Other sexual specializations are also present, such as the presence of modified scales, radial hooks on the fins, and apparently glandular tissues on the caudal fins of males (Malabarba & Weitzman, 1999; 2000; Malabarba et al. 2004; Jerep & Malabarba et al. 2011). Other genera such as *Cheirodon* Girard 1855, *Serrapinnus* Malabarba 1998, and *Heterocheirodon* Malabarba 1998 are characterized by a high number of ventral procurrent caudal fin rays (11 to 30) and by notable secondary sexual specializations of the procurrent ventral caudal fin rays and anal fin rays of males (Campos, 1982; Malabarba, 1998; Malabarba & Bertaco, 1999; Malabarba & Jerep, 2014).

Cheirodontinae species inhabit most Central and South American river drainages, from Costa Rica to central Chile and Argentina, in both the Atlantic and Pacific drainages of the Andes, *Cheirodon* being the genus with the southernmost distribution of the subfamily, and is found in the waters of the western slopes of the Andes in Chile and on the eastern slopes of the Andes in Argentina (Campos, 1982; Malabarba, 2003). Cheirodontinae is a monophyletic subfamily, supported by phylogenetic analysis with morphological (Malabarba, 1998), molecular (Mariguela et al. 2013) and morphological-molecular (Mirande, 2019) data, but the monophyly of some of its genera such as *Compsura* Eigenmann 1915, *Odontostilbe* Cope 1870, and *Serrapinnus* Malabarba 1998

have been questioned in molecular phylogenies (Mariguela et al. 2013). The classification and phylogeny within the Cheirodontinae are still under discussion. The precise delimitation of some species with similar morphological characteristics is a great challenge, additionally because there is a high diversity of species that have to be described.

Throughout history, various methods have been applied to study the species of Cheirodontinae. An initial statistical approach uses computer algorithms that allowed delimiting species with morphological and meristic characters, considering as a criterion that the phenotype of a species represented a normal distribution (Malabarba & Bertaco, 1999; Bührnheim & Malabarba, 2006; 2007; Jerep & Malabarba, 2011; Malabarba & Jerep, 2014; Chuctaya et al. 2018). The limitation of this type of analysis, however, is that it does not allow to solve the problem of convergent evolution. On another approach, the multivariate analyzes allowed to form individual operative taxonomic units (OTU) that shared distinctive characteristics, but these analyzes did not provide the limits of the species, and they did not can identify sources of morphological variation (genetic or environmental origin) (Rannala & Yang, 2020).

With the advancement of molecular studies, we entered molecular taxonomy, the use of molecular data, such as the use of multilocus sequences, and the coalescence theory led several research groups to create species delimitation methodologies (e.g. reciprocal monophyly) (Rannala & Yang, 2020), that are recently being used in Cheirodontinae, testing phylogeographic hypotheses (Mariguela et al. 2011), phylogenetic relationships (Mariguela et al. 2013), and species description (Chuctaya et al. 2020). The molecular approach uses DNA sequences that are grouped into Molecular Taxonomic Units (MOTU), defined as the grouping of individual DNA sequences based on an explicit algorithm used to estimate diversity at the species level (Cañedo-Apolaya et al. 2021).

Species delimitation is the process by which the limits of species are identified and determining which groups of individual organisms constitute different populations of a single species and which constitute different species (Rannala & Yang, 2020), allowing also the discovery of new species. This is a permanent topic in systematics and has been the subject of continuous discussion, from the concept of species (de Queiroz, 2007), differentiate integrative taxonomy from interactive taxonomy (Yeates et al, 2011); and even discuss what are the main errors when delimiting a species (Carstens et al. 2013). In recent years, the field has witnessed a dramatic increase in the number of methods available to delimit species, especially with the advent of new approaches and methods that allow the recognition of geographically isolated populations as independent evolutionary lineages, posing a major problem, that of deciding whether a lineage corresponds to different species or to genetically structured populations (Malabarba et al. 2021).

Hebert et al. (2003) published a proposal for the "DNA barcoding" initiative using a single locus (the COII mitochondrial gene for animals) as a diagnostic for species designation and with this, encouraged the creation of new high-throughput methodologies available to delimit species, such as: ABGD, the Automatic Barcode Gap Discovery (Puillandre et al. 2012), LocMin, a threshold distance based method (Brown et al., 2012), GMYC, the Generalized Mixed Yule Coalescent method (Fujisawa & Barraclough, 2013), PTP, Poisson tree processes (Zhang et al. 2013), mPTP, the multi-rate Poisson tree process method (Kapli et al., 2017), and ASAP, Assemble Species by Automatic Partitioning (Puillandre et al. 2021).

While DNA-based methods are creating an opportunity to speed up the process of putative species delimitation (MOTU), single-locus data provide a practical and rapid approach in studying large numbers of species to generate species hypotheses primary or

candidate species (Garcia-Melo et al. 2019), which will later be complemented with an integrated or interactive taxonomic approach, to finish with its formal description of the species, that currently the general consensus among taxonomists (Garcia-Melo et al. 2019, Malabarba et al. 2021), including many lines of evidence (morphological and molecular), based on the life history of the taxa – on the traits that can be more prone to differentiation – and on the biogeographic evidence of long-term isolation in different river drainages (Malabarba et al. 2021).

Due to known taxonomic uncertainties within the subfamily Cheirodontinae, we obtained a COI sequence data set for the species of the Cheirodontinae from Neotropical Region, with the aim (1) of investigation lineage diversity (MOTU), and (2) to identify species complexes and their distribution patterns.

Material and methods

Taxon sampling

A total of 84 specimens were sequenced in the study, representing 35 morphospecies from collections made in Brazil, Peru, Ecuador. 204 sequences were added to the alignment from Genbank and Barcode of Life Data (BOLD), completing 288 specimens analyzed, from the entire distribution area of the subfamily (**Table S1**). The collected samples were deposited in the fish collection at the Federal University of Rio Grande do Sul (UFRGS) in Brazil and the fish collection of the Natural History Museum of the UNMSM in Peru (MUSM). For each sample, muscle tissue was collected and preserved in 96° ethanol for analysis in the laboratory. Voucher specimens were preserved in 10% formaldehyde and subsequently transferred to 70° ethanol for storage and subsequent morphological analysis.

Morphological identification

For the morphological identification of Cheirodontinae individuals, original descriptions, identification key and phylogenetic analyzes were used (Fink & Weitzman, 1974; Campos, 1982; Malabarba,1998; Malabarba & Bertaco, 1999; Malabarba & Weitzman, 1999; 2000; Bührnheim & Malabarba, 2006; 2007; Jerep & Malabarba 2011; Malabarba & Jerep, 2014; Chuctaya et al. 2018). For individuals that could not be identified to the species level using the taxonomic reviews, an identification code was used.

DNA extraction and Sequencing

DNA extraction from an ethanol-preserved tissue sample followed the method of Brometo de Cetil-Trimetil- Amônio (CTAB) (Doyle & Doyle 1987). Amplifications by Polymerase Chain Reaction (PCR) were carried out following the conditions of Thomaz et al. (2015), using the primers FishF1 and FishR1 (Ward et al., 2005), L6252 and H7271 (Melo et al. 2011). The PCR product was bidirectional sequenced at Macrogen Inc. (South Korea) using a high throughput Applied Biosystems 3037 XL technology. The organization, verification and edition of the sequences was carried out in the software Geneious 8.1.4 (Drummond et al., 2010). The chromatogram reads for each sample sequenced were assembled into contigs and verified visually. We also translated the contigs into putative amino acids to check for the presence of stop codons; no internal stop codons were found in the alignment.

Alignment and phylogenetic analyses

Sequences were aligned using MUSCLE algorithm (Edgar, 2004) implemented in the Mega X software (Kumar et al., 2018) using Cluster Method (interaction 1,2) UPGMA (Unweighted Pair-Group Method using Arithmetic Averages) and under default

parameters. *Aphyocharax anisitsi* Eigenmann & Kennedy 1903 and *Aphyocharax dentatus* Eigenmann & Kennedy 1903 representing the subfamily Aphyocharacinae, *Rachoviscus crassiceps* Myers 1926 and *Cheirodon jaguaribensis* Fowler 1941 of the subfamily Stethaprioninae, were used as outgroup species based on the results obtained from the phylogenetic relationship analyzes of Mariguela et al. (2013) and Mirande (2019). A Neighbor-Joining tree containing all sequences is provided as Supplemental material (Fig. S1). All new sequences generated in this studio will be available in GENBANK and BOLD. Information on the individuals analyzed in this study is presented in **Table S1.**

Species delimitation

Phylogenetic relationships were inferred by Bayesian Inference using the software BEAST 2.6.2 (Bouckaert et al., 2019), according to the following settings: nucleotide substitution model using the general time reversible model with among-site rate heterogeneity GTR + I + G (Gu et al., 1995) as the best-fit evolutionary model estimated in jModelTest2 (Darriba et al., 2012); single site model partition; strict molecular clock; Yule model tree prior. We run a search with 100 million generation of Markov Chain Monte Carlo (MCMC), sampling tree topologies and parameters every 10000 generations. The convergence of parameters of each run was observed by checking the values of effective sample size (ESS > 200) and stationarity of the chain using the software TRACER 1.7.1 (Rambaut et al., 2018). The production of a maximum credibility tree was using TREEANNOTATOR (Bouckaert et al., 2019).

Additionally, we generate the best maximum likelihood (ML) tree, the RAxML was run in RAxML-HPC2 on XSEDE 8.2.9 (Stamatakis, 2006; Stamatakis et al., 2008) via CIPRES portal v3.3 (Miller et al., 2010), RAxML searches were conducted using 10

parallel runs, starting with a randomly generated tree. Branch support was assessed using the rapid bootstrap algorithm with 1000 replicates.

Species delimitation approaches involved seven methods: (a) ABGD, the Automatic Barcode Gap Discovery (Puillandre et al. 2012); (b) LocMin, a threshold distance based method (Brown et al., 2012); (c) ASAP, Assemble Species by Automatic Partitioning (Puillandre et al. 2021); (d) GMYC, the Generalized Mixed Yule Coalescent method (Fujisawa & Barraclough, 2013); (e) PTP, Poisson tree processes (Zhang et al. 2013); (f) bPTP the Bayesian Poisson Tree Processes (Zhang et al. 2013); and (g) mPTP, the multi-rate Poisson tree process method (Kapli et al., 2017).

We used the aligned sequences generated in Muscle for the following analyzes: For ABDG, as input file at the ABGD webserver (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) under default parameters; for LocMin, we used a p-distance based method using the 'locMin' and 'tclust' functions, a distance threshold optimization and a clustering approach implemented in SPIDER (Brown et al., 2012) out in the R statistical software v. 3.6.2 (R Development Core Team, 2011); for ASAP, as input file at the ASAP webserver (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) under default parameters.

We used the maximum credibility tree as input of three single-locus species delimitation analysis: For GMYC, we used GMYC webserver (https://species.h-its.org/gmyc/) with single threshold method. For PTP and bPTP, we used PTP webserver (https://species.h-its.org/ptp/) under default parameters.

We used the best maximum likelihood tree as input for the following species delimitation analyses: PTP and mPTP webserver (https://mptp.h-its.org/#/tree) under default parameters.

Mapping species distribution

To understand the distribution of Cheirodontinae lineages in the Neotropical region, we compared lineage diversity with the biogeographical units proposed by Abell et al. (2008), and with the units proposed by Dagosta & De Pinna (2017) for the Amazon basin. This information was inserted into the names of the terminals in the species delimitation tree. We used the results of the different species delimitation methods and analyze the distribution of species that contradict the morphological identification.

Results

We obtained partial COI sequences of Cheirodontinae from 84 specimens, representing 35 morphospecies, distributed in 19 freshwater biogeographic units for the Neotropical region, the addition of data from Genbank and BOLD increased this data set to 288 specimens, 51 morphospecies and 34 biogeographical units. The analysis of the morphospecies using the morphological and molecular information, allowed correcting the identification of 95 specimens deposited in the Genbank and BOLD database. Several of these species were identified up to the level of the order Characiformes (e.g. *Cheirodon interruptus*, *Odontostilbe microcephala*, *Odontostilbe paraguayensis*, *Odontostilbe pequira*, *Serrapinnus calliurus*, *Serrapinnus kriegi*); other species were identified only up to subfamily and genus level. These corrections are presented in **Table S2.**

Sequence length ranged from 298 to 531 bp, with a mean length of 524 bp. Of the aligned sequences, 197 sites were informative for parsimony, 209 sites were variable, and 322 sites were conserved. Fifty one morphospecies were analyzed (38 valid species, 4 morphospecies that need a larger number of samples to verify their identification and 9 morphospecies with different morphology and not described). The list of morphospecies analyzed as well as information on localities, and collection codes are presented in **Table**

S1. The number of individuals per morphospecies varied from 1 to 48, the number of biogeographical units for each morphospecies varied from 1 to 5 units, *Cheirodon interruptus* being the species with the largest number of individuals analyzed and biogeographical units. The intraspecific distance varied from 0 to 0.026. The interspecific distance varied from 0.006 to 0.263. The species of the genus *Cheirodon* present the smallest interspecific distance (**Table 1**). The number of MOTUs resulting from the analysis with the different species delimitation methods were 53 (ABDG), 53 (ASAP), 68 (LocMin), 53 (GYMC), 55 (PTP-1 with UM tree), 59 (bPTP with UM tree), 49 (PTP-2 with RXML tree) and 35 (mPTP with RXML tree) (**Table 2**).

The most conservative methods were PTP-2 and mPTP. These methods combined species with highly differentiated morphological characteristics into a single MOTU. PTP-2 considered the species *Serrapinnus heterodon* and *Odontostilbe stenodon* as a single MOTU, while mPTP considered the species *Serrapinnus notomelas*, *S. calliurus*, Snotm, and *Serrapinnus* sp2. as a single MOTU, additionally together *Odontostilbe pequira*, *Serrapinnus heterodon*, *Odontostilbe stenodon* and *Odontostilbe pequira*-Opeur as a single MOTU, and *Odontostilbe dialeptura*, *O. mitoptera*, *Compsura gorgonae* and Odchi as another single MOTU (Fig. 1, Fig. 2, and Fig. 3). The methods ABDG, ASAP, GYMC, LocMin, PTP-1 and bPTP, presented similar results, coinciding in the species delimitations of the following MOTUs: *Acinocheirodon melanogramma*, *Aphyocheirodon hemigrammus*, *Cheirodon ibicuhiensis*, *Cheirodontops geayi*, *Compsura gorgonae*, *C. heterura*, *Heterocheirodon jacuiensis*, *Kolpotocheirodon theloura*, *Odontostilbe pequira* Opeur, Ossp , Smdd, Obur, Ochi, Ctko, Prodonsp, Pger, *Odontostilbe microcephala*, *O. pacaasnovos*, *O. splendida*, *O. pequira*-morpho1, *O. mitoptera*, *O. dialeptura*, *Pseudocheirodon arnoldi*, *P. terrabae*, , *Prodontocharax* sp,, *Prodontocharax alleni*, *Protocheirodon pi*-morpho1, *Serrapinnus notomelas*-morpho1,

S. notomelas-morpho2, *S. calliurus*, *S. gracilis*, *S. kriegi*, and *S. heterodon*. Six morphospecies were recognized for a singleton: *Cheirodon* cf. *galusdae*, *Cheirodon kiliani*, *Ctenocheirodon pristis*, *Heterocheirodon yatai*, *Macropsobrycon* cf. *uruguayanae*, *Serrapinnus* sp2 (**Table 1**), and as a result of the analysis *Odontostilbe pequira*-08 and *Serrapinnus heterodon*-03 (Fig. 1), requiring a greater number of samples as well as complementary analyses to verify the taxonomic status of these MOTUs.

Odontostilbe fugitiva and *O. nareuda* were recognized as a single MOTUS by most methods of species delimitation, except for bPTP which considered these two species as different MOTUs. *Cheirodon interruptus* and *C. australis* were also recognized as a single MOTU by most methods, except for LocMin, *Cheirodon pisciculus* and *C. galusdae* too were only recognized as different MOTUs by the LocMin method. Some species presented several MOTUS for the different methods applied (ABDG, GYMC, LocMin, ASAP, PTP, bPTP) such as *Macropsobrycon uruguayanae* (2), *Odontostilbe pequira* (2), *O. euspilurus* (2), *Protocheirodon pi* (3), *Serrapinnus heterodon* (2), and *S. notomelas* (2), (**Table 1**).

Discussion

The phylogenetic tree does not have the objective of resolving or proposing new phylogenetic relationships, results that would only be obtained after an integrative analysis with morphological and multilocus molecular information. Our objective is to recognize possible new species in fish collections using the COI molecular marker together with species delimitation methodologies. These methods allow the recognition of MOTUs, which in some cases correspond to unreported species that are part of cryptic species, or that share a morphological similarity that makes it difficult to identify them with a single (morphological) evidence, going unnoticed since their first collections.

These candidate species would only be valid after a formal description, with an integrative morphological and molecular analysis, in many cases with a perspective based on the life history of the taxa – on the traits that can be more prone to differentiation – and on the biogeographic evidence of long-term isolation in different river drainages (Malabarba et al. 2021).

Species delimitation is the act of identifying biological diversity at the species level. In recent years, the field has witnessed a dramatic increase in the number of methods available to delimit species (Carstens et al. 2013). However, the most recent published researches only used a few methods (i.e., 2 or 3) of the total available methods, often for unknown reasons, from methods using genetic distance such as LocMin, ABDG, ASAP (Puillandre et al. 2012; Brown et al., 2012; Puillandre et al. 2021) as well as methods that depend on an ultrametric tree such as GMYC (Fujisawa & Barraclough, 2013), and methods that only need a rooted phylogenetic tree such as PTP, bPTP, and mPTP (Zhang et al., 2013; Kapli et al., 2017). Each of these methods makes a series of simplifying assumptions that could be violated in each of the particular analyses. To know how our species, respond to all these methods, we tested a wide range of these methods, with the aim of knowing the congruence between them, as it was observed in figure 1, the ABDG, ASAP, GMYC, PTP and bPTP methods. present consistent results. In any case, these inferences will be considered conservatively, until an analysis with several lines of evidence is carried out, since in most contexts it is better not to consider a MOTU as a species, until corroborating that it represents a real evolutionary lineage.

knowing an unknown diversity

This study included 38 valid species (58% of the total known species) with representatives in 34 biogeographical units. In addition, after a morphological analysis, we identified nine candidate species for new species (Ctko, Obur, Odchi, Opeur, Ospp,

Pger, Prodosp, Smdd, and Snotm), a result that was corroborated by most of the species delimitation methods used in this study (Fig. 1). For some of these candidate species it was not possible to assign a valid genus, as they did not present the diagnostic characteristics of the valid genera within the Cheirodontinae described by Malabarba (1998), Malabarba & Weitzman (1999, 2000), Malabarba & Jerep (2012), and Vari et al. (2016), requiring a more comprehensive analysis of phylogenetic relationships in the subfamily.

The species delimitation analysis shows a still unknown high diversity within Cheirodontinae. The most conservative method (mPTP) indicates 35 MOTUs, and the least conservative (LocMin) 68 MOTUs. The results of mPTP depend on the branch length and number of samples, as it is observed in *Cheirodon*, whose species present short branches, representing a recent diversification. In the analysis with mPTP, the results show the union of a Cis-Andean species (*Cheirodon australis*) and a trans-Andean species (*Cheirodon interruptus*) as a single MOTU. These species, however, have very separate distributions (Malabarba 2003), distinctive morphologies (Campos 1982), as well as chromosomal characteristics (Soto et al. 2018) that easily separate and diagnose each other, contradicting the mPTP result. Similar case was observed with *Serrapinnus heterodon*, *Odontostilbe pequira*, and *Odontostilbe stenodon* that present remarkable diagnostic characteristics that allow diagnosing the species (Malabarba 1998) and that were recognized by this methodology as a single MOTU. In the case of LocMin, a method based on the threshold distance (Brown et al. 2012), it is influenced by the minimum interspecific distance, this value being low, as was observed among *Cheirodon* species (**Table 2**).

Among the species that present several MOTUs we find *Serrapinnus notomelas*, that presents two MOTUs, corroborated by the methods (ABDG, ASAP, LocMin,

GMYC, PTP-1, bPTP). This species was previously studied by Mariguela et al. (2011) who determined the presence of three populations within this species, suggesting that these populations may be on independent evolutionary trajectories, which represents a very early stage of diversification in these populations. The results of Mariguela et al. (2011) combined with the analysis of species delimitation, demonstrate the presence of two MOTUs, which need to be evaluated using integrative methods and solve the population-species dilemma, which implies deciding whether to consider separate lineages as different species or structured genetic populations (Malabarba et al. 2021)

Odontostilbe pequira presents three MOTUs, two (*Odontostilbe pequira* and *O. pequira*-Opeur) strongly differentiated by 7 methods ABDG, ASAP, LocMin, GMYC, PTP-1, bPTP, PTP-2). The MOTU *O. pequira*-Opeur is distributed mainly in the Uruguay River Basin and in the lower part of the Lower Paraná Basin, while the MOTU *Odontostilbe pequira* is distributed in the Paraguay River Basin, and in the upper parts of the Lower Paraná Basin. Preliminary morphological results of these two MOTUs show slight differences in the coloration pattern of the spot on the dorsal fin. An integrative study is necessary to determine the taxonomic status of this species. The MOTU *Odontostilbe pequira*-08, which is distributed in the Paraguay River Basin, is a singleton and requires a greater number of samples to obtain more information about the taxonomic status of this MOTU.

Macropsobrycon uruguayanae presents two MOTUs for five methods used (GMYC, LocMin, PTP-1, bPTP, PTP-2) (Fig. 1). This species is currently monotypic, and was redescribed by Jerep & Malabarba (2011), who compared the populations between the Uruguay River, Negro River and Laguna dos Patos, where this species is distributed. Jerep & Malabarba (2011), in their analysis, found slight morphological differences between these populations, differences that were not corroborated with their

morphometric analysis. The Laguna dos Patos basin specimens, presented somewhat larger caudal peduncle length and upper jaw length, and relatively smaller anal-fin base length than specimens from Uruguay river basin. In the river system comprising the Uruguay river and Negro river drainages (i.e., the La Plata basin) vs. the Laguna dos Patos (in some cases extended to the Tramandaí river drainage), there are several pairs of geographically isolated and morphologically diagnosed sister species as exemplified by *Heterocheirodon yatai* (Casciotta, Miquelarena and Protogino, 1992) vs. *Heterocheirodon jacuiensis* Malabarba & Bertaco, 1999; *Lepthoplosternum pectorale* (Boulenger, 1895) vs. *Lepthoplosternum tordilho* Reis, 1997; *Oligosarcus oligolepis* (Steindachner, 1867) vs. *Oligosarcus robustus* Menezes, 1969; *Otocinclus arnoldi* Regan, 1909, vs. *Otocinclus flexilis* Cope, 1894; *Parapimelodus valenciennis* (Lütken 1874) vs. *Parapimelodus nigribarbis* (Boulenger, 1889) and *Bunocephalus doriae* Boulenger, 1902 vs. *Bunocephalus erondinae* Cardoso, 2010; *Pseudocorynopoma doriae* Perugia, 1891 vs. *Pseudocorynopoma stanleyi* Malabarba, Chuctaya, Hirschmann, Oliveira & Thomaz 2020 (Cardoso, 2010; Lehmann et al., 2010; Lucena et al., 1992; Malabarba & Bertaco, 1999; Reis, 1997; Wendt et al., 2019; Malabarba et al. 2021). In the case of *Macropsobrycon uruguayanae*, we need to apply an integrative approach using both comparative morphology and molecular phylogeographic methods to address the species–population dilemma: do *M. uruguayanae* populations correspond to historically structured lineages or to different species? similar to that realized by Malabarba et al. (2021). In our analysis we find *Macropsobrycon* cf. *uruguayanae*, as a MOTU different from *M. uruguayanae* resulting from the application of 7 species delimitation methods (Fig. 1). This MOTU is distributed in the Upper Paraná biogeographical unit, outside the currently known distribution area of *M. uruguayanae*

(Jerep & Malabarba, 2011), *Macropsobrycon* cf. *uruguayanae* needs of a greater number of samples and analysis, which would allow us to corroborate its taxonomic status.

Misrepresented diversity

Ideally, all barcode sequences contained in either database (Genbank and BOLD) should have been derived from a vouchered specimen, which was initially identified by a taxonomic expert. However, given the inherent nature of any public database, it is inevitable that some erroneous data will be present. The generation and submission of incorrect sequences likely occurs due to misidentification of the original material, poor isolation techniques, contamination, duplicate records due to instances of synonymy and PCR-based errors (Meiklejohn et al. 2019). Currently there are few studies that have evaluated the accuracy of the sequence data contained in Genbank and BOLD. Bridge et al. (2003) and Nilsson et al. (2006) studied this divergence in fungi, Jin et al. (2020) in algae, and Seah et al. (2017) studied this divergence with COI in fish (Leiognathidae: Acanthuriformes). Here, a total of 209 sequences of 51 morphospecies from subfamily Cheirodontinae has been downloaded from these public databases. From all the sequences that have been downloaded, a total of 95 sequences (45%) has been detected as potential misidentification or imprecise identification at a higher taxonomic level (order, family, subfamily, genus), as these sequences did not group with their own taxa. There are six species (*Cheirodon interruptus*, *Odontostilbe microcephala*, *O. pequira*, *O. paraguayensis*, *Serrapinnus calliurus* and *S. kriegi*) that had been assigned to only as order Characiformes. This identification error of 45% of deposited COI sequences, shows that for Cheirodontinae there is a precision and reliability of 55% for the species identified in the public databases and it is manifest that the greatest challenges to the used biological barcoding to Cheirodontinae will be of taxonomical nature, rather than technical. Although Genbank and BOLD Systems have been established as public sequence

libraries, the accuracy of deposited sequences should be monitored to ensure the success of species identification (Seah et al. 2017).

Conclusions

The results of the single-locus species delimitation methods complemented the previously performed morphological delimitations; the nine morphospecies identified in this study (Ctko, Obur, Odchi, Opeur, Ospp, Pger, Prodosp, Smdd, and Snotm) were also delimited as MOTUs by most of the applied methods. All the species described, with the exception of *Odontostilbe fugitiva*, *O. nareuda*, *Cheirodon australe*, *C. pisciculus* and *C. galusdae*, were not recognized as independent MOTUs by most of the methods, requiring the application of complementary analyzes to determine the taxonomic status of these species, and four species *Serrapinnus notomelas*, *Odontostilbe pequira*, *Protocheirodon pi* and *Macropsobrycon uruguayanae*, present several MOTUs, corroborated by most of the applied methods. Therefore, these results provide strong and consistent evidence for additional taxonomic diversity in Cheirodontinae. Although not formally described in this study, these nine MOTUs (Ctko, Obur, Odchi, Opeur, Ospp, Pger, Prodosp, Smdd, and Snotm) increase the taxonomic diversity of the Cheirodontinae by 14%. Our sampling was not exhaustive, however, and we expect additional species to be discovered mainly in under-sampled genera not included in this study such as *Saccoderma* and *Nanocheirodon,* as well as from poorly-sampled regions such as the Amazon Basin, Orinoco, and Magdalena.

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FIGURES

Figure 1. Maximum clade credibility chronogram using BEAST 2.6. Dataset comprised 288 sequences of Cheirodontinae COI sequences. Species delimitations are shown by method as colored boxes. In each sequence, the basin from which the sample comes is indicated. Part 1.

Figure 2. Maximum clade credibility chronogram using BEAST 2.6. Dataset comprised 288 sequences of Cheirodontinae COI sequences. Species delimitations are shown by method as colored boxes. In each sequence, the basin from which the sample comes is indicated. Part 2.

Figure 3. Maximum clade credibility chronogram using BEAST 2.6. Dataset comprised 288 sequences of Cheirodontinae COI sequences. Species delimitations are shown by method as colored boxes. In each sequence, the basin from which the sample comes is indicated. Part 3.

TABLES

Table 1. Summary statistics of the morphospecies analyzed in this study, including nominal species and candidate species of Cheirodontinae.

SUPPLEMENTAL INFORMATION

Table S1. Name, species code, museum voucher numbers, GenBank accession numbers, basin where the sample was collected, for all Cheirodontinae individuals used in this study

Table S2: List of specimens of Cheirodontinae deposited in GenBank and BOLD, whose names were corrected or updated during this study.

Figure 1S. Neighbor joining phylogenetic tree showing all 337 COI sequences of Cheirodontinae, constructed from p-distance. Part 1

Figure 1S. Neighbor joining phylogenetic tree showing all 337 COI sequences of Cheirodontinae, constructed from p-distance. Part 2

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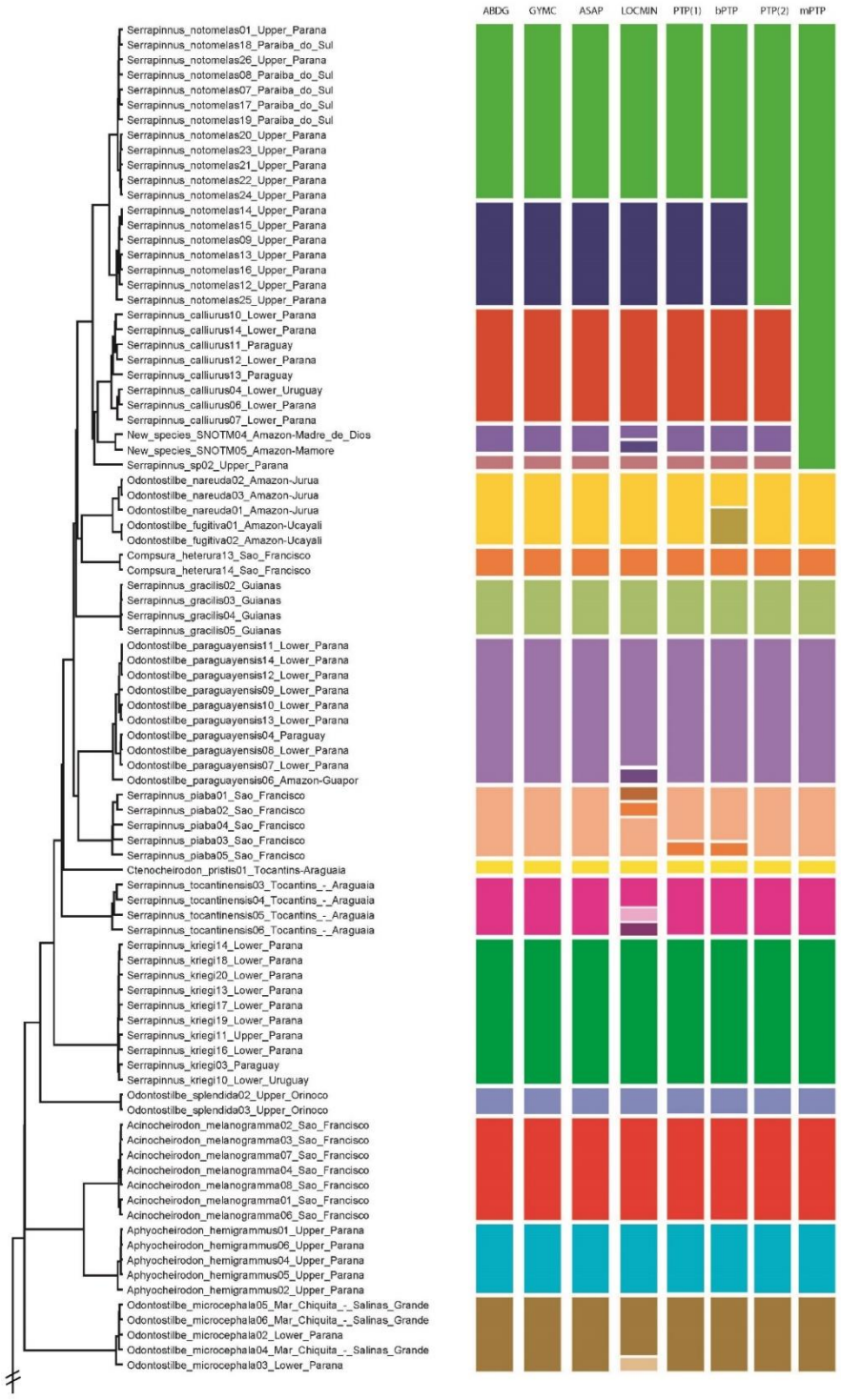
Table 1. Summary statistics of the morphospecies analyzed in this study, including nominal species and candidate species of Cheirodontinae.

N	Species nominales	Ind	Basin	Mean Intra Dist	Min Mean Inter Dist	Monophyly	ABDG	GYMC	ASAP	LocMim	PTP-1	bPTP	PTP-2	mPTP
1	*Aphyocheirodon hemigrammus*	5	1	0.002	0.065	True	1	1	1	1	1	1	1	1
2	*Acinocheirodon melanogramma*	7	1	0.001	0.107	True	1	1	1	1	1	1	1	1
3	*Cheirodon australe*	4	1	0.000	0.011	True	0	0	0	1	0	0	0	0
4	*Cheirodon cf. galusdae*	1	1	0.000	0.006	Singleton	1	1	1	1	1	1	0	0
5	*Cheirodon galusdae*	2	1	0.004	0.010	True	0	0	0	1	0	0	0	0
6	*Cheirodontops geayi*	5	2	0.011	0.089	True	1	1	1	1	1	1	1	1
7	*Compsura gorgonae*	7	2	0.007	0.038	True	1	1	1	2	1	1	1	0
8	*Compsura heterura*	2	1	0.004	0.068	True	1	1	1	1	1	1	1	1
9	*Cheirodon ibicuhiensis*	6	2	0.003	0.061	True	1	1	1	1	1	1	1	1
10	*Cheirodon interruptus*	48	5	0.003	0.027	True	0	0	0	1	0	0	0	0
11	*Cheirodon kiliani*	1	1	0.000	0.018	Singleton	1	1	1	1	1	1	0	0
12	*Cheirodon pisciculus*	3	1	0.000	0.076	True	0	0	0	1	0	0	0	0
13	*Ctenocheirodon pristis*	1	1	0.000	0.088	Singleton	1	1	1	1	1	1	1	1
14	*Species CTKO*	2	1	0.000	0.124	True	1	1	1	1	1	1	1	0
15	*Heterocheirodon jacuiensis*	3	1	0.010	0.025	True	1	1	1	2	1	1	1	0
16	*Heterocheirodon yatai*	1	1	0.000	0.094	Singleton	1	1	1	1	1	1	1	0
17	*Kolpotocheirodon theloura*	5	2	0.001	0.093	True	1	1	1	1	1	1	1	1
18	*Macropsobrycon uruguayanae*	3	2	0.026	0.095	True	1	2	1	2	2	2	2	0
19	*Macropsobrycon cf. uruguayanae*	1	1	0.000	0.113	Singleton	1	1	1	1	1	1	1	0
20	*Species Obur*	5	2	0.001	0.099	True	1	1	1	1	1	1	1	1
21	*Species Odchi*	2	1	0.008	0.036	True	1	1	1	1	1	1	1	0
22	*Odontostilbe dialeptura*	3	1	0.003	0.054	True	1	1	1	2	1	1	1	0
23	*Odontostilbe euspilurus*	2	2	0.017	0.096	True	2	1	2	2	2	2	1	1

N	Species nominales	Ind	Basin	Mean Intra Dist	Min Mean Inter Dist	Monophyly	ABDG	GYMC	ASAP	LocMim	PTP-1	bPTP	PTP-2	mPTP
24	*Odontostilbe fugitiva*	2	1	0.000	0.014	True	0	0	0	0	0	1	0	0
25	*Odontostilbe pacaasnovos*	2	1	0.000	0.059	True	1	1	1	1	1	1	1	1
26	*Odontostilbe microcephala*	5	3	0.005	0.104	True	1	1	1	2	1	1	1	1
27	*Odontostilbe mitoptera*	2	1	0.002	0.119	True	1	1	1	1	1	1	1	0
28	*Odontostilbe nareuda*	3	1	0.004	0.059	True	0	0	0	0	0	1	0	0
29	*Odontostilbe paraguayensis*	10	3	0.007	0.061	True	1	1	1	2	1	1	1	1
30	*Odontostilbe pequira*	15	4	0.009	0.044	True	2	2	2	3	2	2	1	0
31	*Species Opeur01*	13	3	0.003	0.041	True	1	1	1	2	1	2	1	0
32	*Odontostilbe splendida*	2	1	0.002	0.087	True	1	1	1	1	1	1	1	1
33	*Species Ospp*	5	3	0.009	0.068	True	1	1	1	1	1	2	1	1
34	*Odontostilbe stenodon*	5	1	0.008	0.040	True	1	1	1	1	1	2	0	0
35	*Prodontocharax alleni*	4	2	0.000	0.172	True	1	1	1	1	1	1	1	1
36	*Species Pger*	3	1	0.000	0.165	True	1	1	1	1	1	1	1	1
37	*Protocheirodon pi*	8	4	0.012	0.159	True	3	3	3	3	3	3	3	3
38	*Prodontocharax sp.*	3	1	0.001	0.134	True	1	1	1	1	1	1	1	1
39	*Species Prodos*	2	1	0.000	0.166	True	1	1	1	1	1	1	1	0
40	*Pseudocheirodon arnoldi*	5	2	0.003	0.049	True	1	1	1	1	1	1	1	1
41	*Pseudocheirodon terrabae*	2	1	0.000	0.135	True	1	1	1	1	1	1	1	1
42	*Serrapinnus calliurus*	8	3	0.012	0.033	True	1	1	1	1	1	1	1	0
43	*Serrapinnus sp2*	1	1	0.000	0.042	Singleton	1	1	1	1	1	1	1	0
44	*Serrapinnus gracilis*	4	1	0.000	0.066	True	1	1	1	1	1	1	1	1
45	*Serrapinnus heterodon*	23	4	0.008	0.079	True	2	2	2	2	2	2	0	0
46	*Serrapinnus kriegi*	10	4	0.001	0.095	True	1	1	1	1	1	1	1	1
47	*Species Smdd*	2	1	0.010	0.136	True	1	1	1	1	1	1	2	1
48	*Serrapinnus notomelas*	19	2	0.009	0.044	True	2	2	2	2	2	2	0	0

N	Species nominales	Ind	Basin	Mean Intra Dist	Min Mean Inter Dist	Monophyly	ABDG	GYMC	ASAP	LocMim	PTP-1	bPTP	PTP-2	mPTP
49	*Species Snotm*	2	2	0.012	0.066	True	1	1	1	2	1	1	1	0
50	*Serrapinnus piaba*	5	1	0.011	0.057	True	1	1	1	3	2	2	1	1
51	*Serrapinnus tocantinensis*	4	1	0.011	0.263	True	1	1	1	3	1	1	1	1
						Total	53	53	53	68	55	59	49	35



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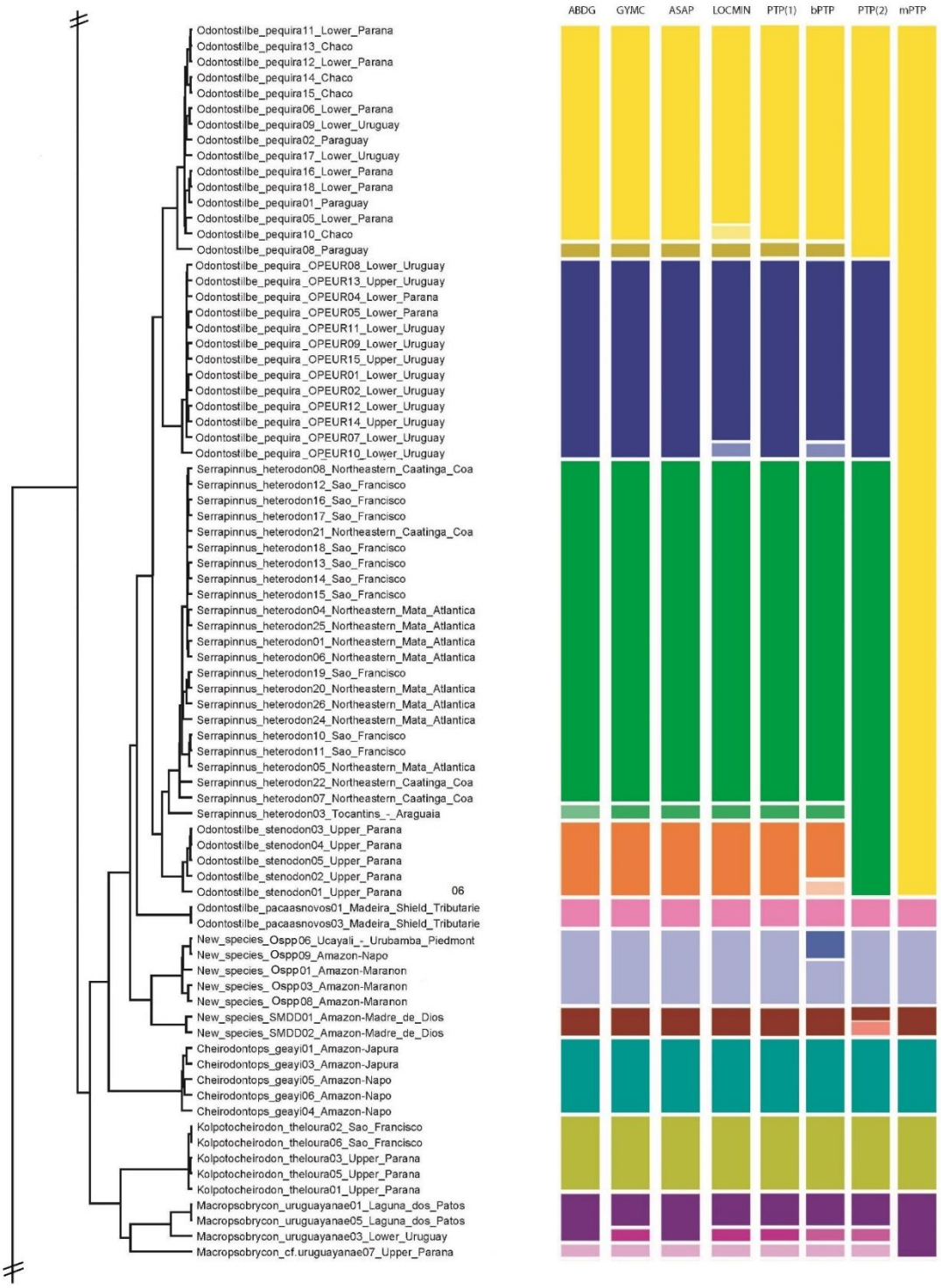

Figure 2. Maximum clade credibility chronogram using BEAST 2.6. Dataset comprised 288 sequences of Cheirodontinae COI sequences. Species delimitations are shown by method as colored boxes. In each sequence, the basin from which the sample comes is indicated. Part 2.

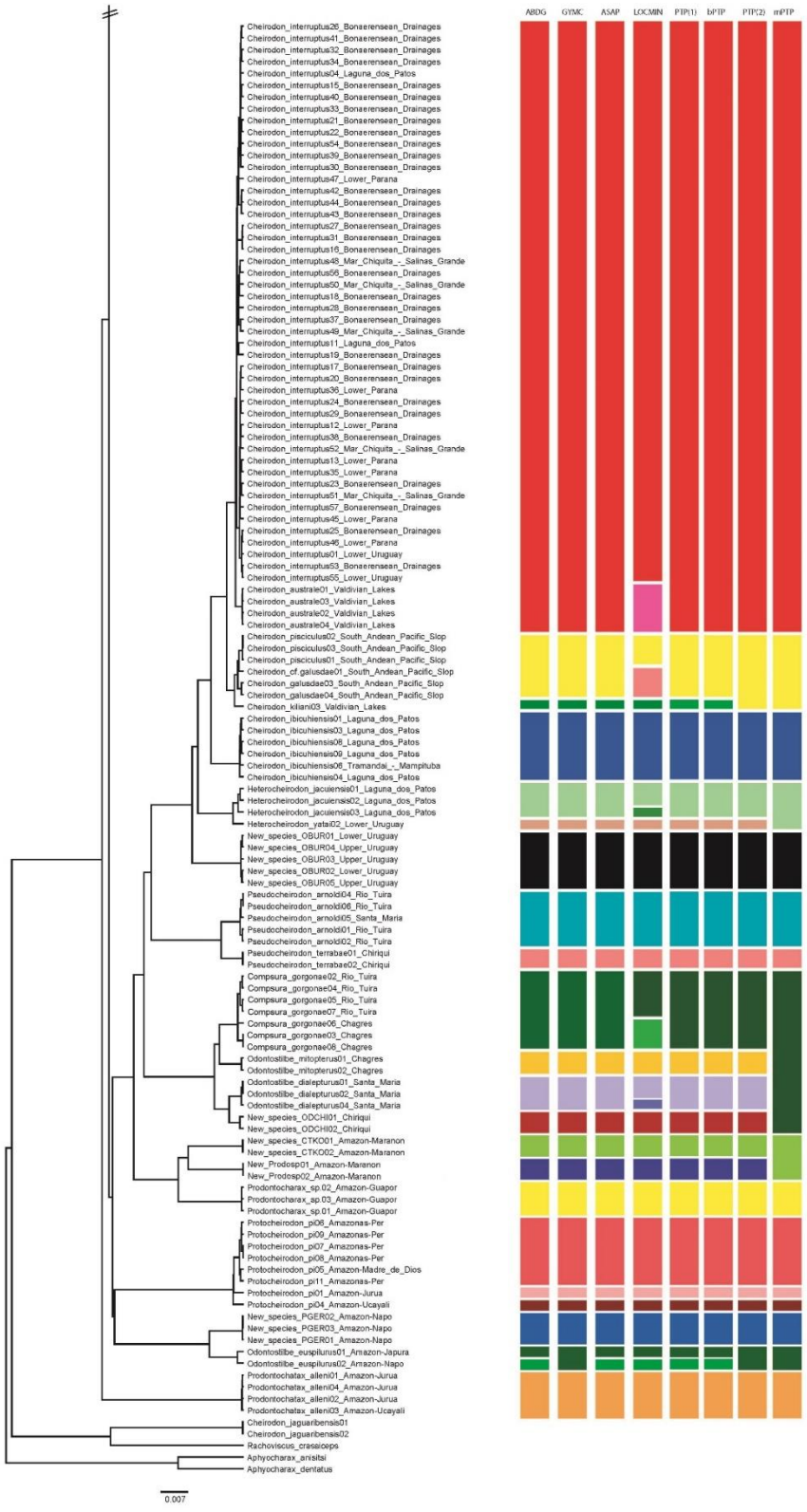

Figure 3. Maximum clade credibility chronogram using BEAST 2.6. Dataset comprised 288 sequences of Cheirodontinae COI sequences. Species delimitations are shown by method as colored boxes. In each sequence, the basin from which the sample comes is indicated. Part 3.

Supplemental Information

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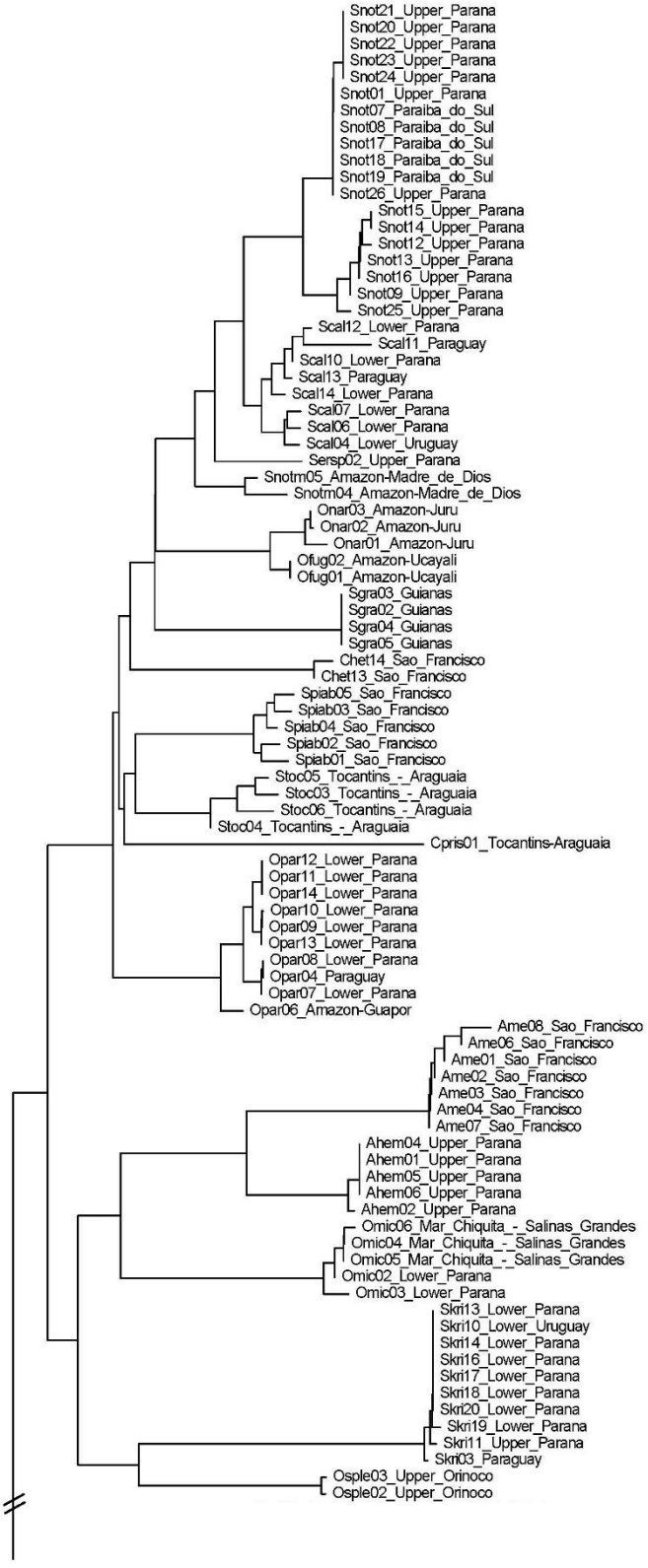
Table S2: List of specimens of Cheirodontinae deposited in GenBank and BOLD, whose names were corrected or updated during this study.



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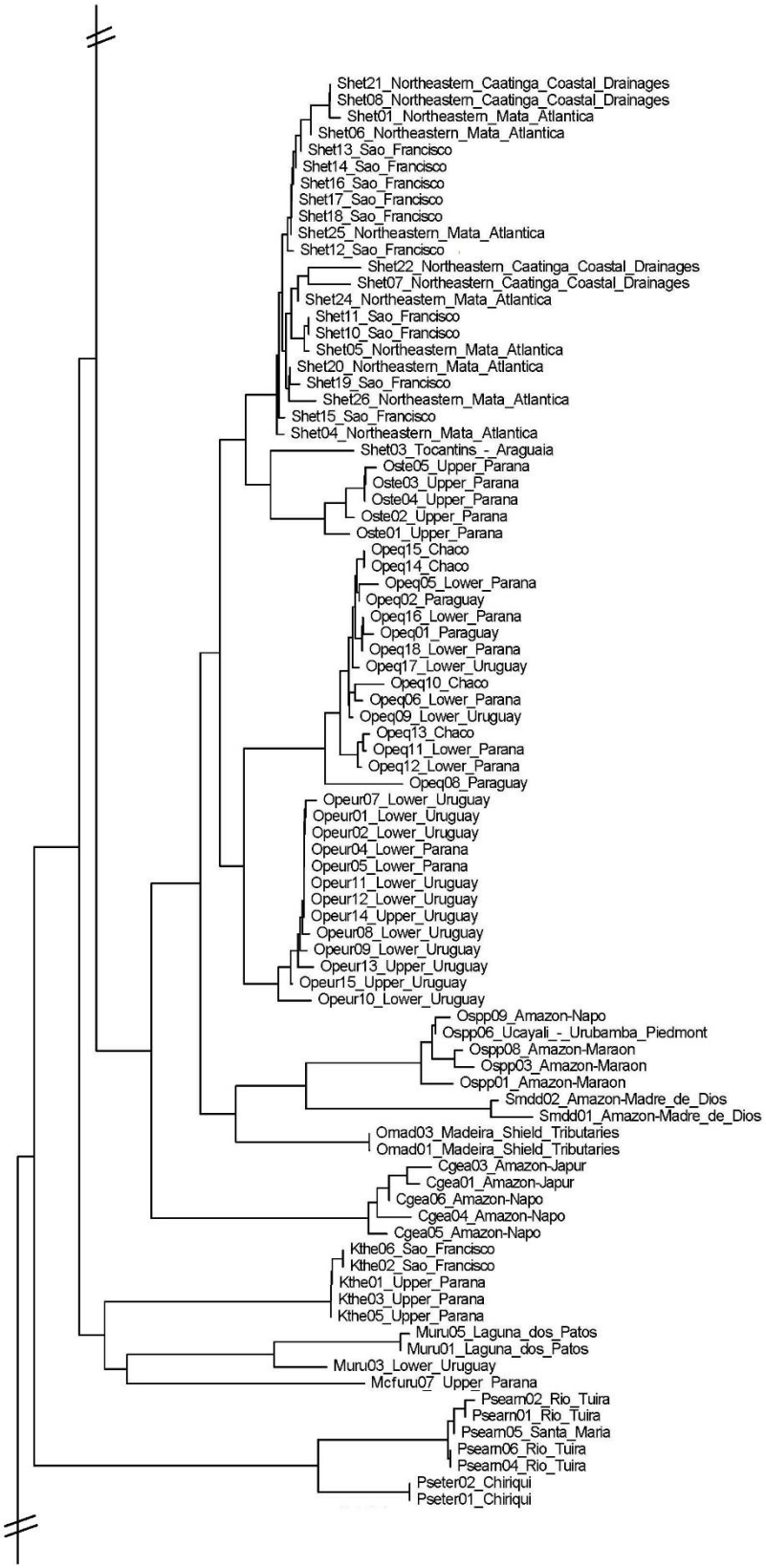

Figure 2S. Neighbor joining phylogenetic tree showing all 337 COI sequences of Cheirodontinae, constructed from p-distance. Part 2

Figure 3S. Neighbor joining phylogenetic tree showing all 337 COI sequences of Cheirodontinae, constructed from p-distance. Part 3

CHAPTER 2

Incongruence between molecules and morphology: A total-evidence phylogeny paves the way for reclassification (Characidae: Cheirodontinae)

Incongruence between molecules and morphology: A total-evidence phylogeny paves the way for reclassification (Characidae: Cheirodontinae)

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ABSTRACT

Molecular and morphological research often suggest conflicting results. Selective pressure on certain morphologies can confound understanding of evolutionary relationships. Cheirodontinae is one of the most diverse subfamilies of Characidae and contains 66 valid species. In addition, the subfamily has one of the widest geographical distributions in the family, being found in a great variety of environments of the Neotropical region. Currently, there are two proposals of classification in tribes within the subfamily: Cheirodontini, Compsurini and Incertae-sedis based on morphological analysis; and Protocheirodontini, Pseudocheirodontini, Cheirodontini and Compsurini based on molecular analysis. The purpose of this contribution is to determine the phylogenetic relationships of the Cheirodontinae members combining morphological and molecular data with Maximum Parsimony analysis and evaluate these classification schemes. A total of 267 morphological characters, six molecular markers and 79 taxa are analyzed, of which 65 belong to Cheirodontinae (57 valid species, and 8 undescribed species). According to our results, Cheirodontinae is divided into 8 main clades supported by the different methods applied (molecular and total evidence), that must be considered tribes in order to maintain the tribe names previously proposed as monophyletic: Protocheirodontini, Amblystilbini, Pseudocheirodontini, Prodontocharacini, Cheirodontini, Holoshesthini, Macropsobryconini and Compsurini. *Odontostilbe* and *Serrapinnus* are not monophyletic. We recovered species attributed to *Odontostilbe* in different clades (Pseudocheirodontini, Prodontocharacini, Holoshesthini and Compsurini), likewise *Serrapinnus* was found in the clades Holoshesthini and Compsurini. Within Protocheirodontini we find the type species *Protocheirodon pi*; in Amblystilbini *Amblystilbe alleni*; in Pseudocheirodontini, *Pseudocheirodon*, *Nanocheirodon*, and two new genera; in Prodontocharacini, *Prodontocharax melanotus*,

Saccoderma, "*Odontostilbe" mitoptera*, "*O". dialeptura*, "*Compsura" gorgonae* and a new genus; in Cheirodontini, *Cheirodon*, *Heterocheirodon* and a new genus; in Holoshesthini, *Acinocheirodon*, *Aphyocheirodon*, *Cheirodontops*, and the resurrected *Holoshesthes*; in Macropsobryconini, *Macropsobrycon* and *Kolpotocheirodon*; and in Compsurini, *Serrapinnus* sensu stricto, *Odontostilbe* sensu stricto, *Compsura*, *Ctenocheirodon*, clade G1, clade G2 and the fossil species *Megacheirodon unicus*+.

INTRODUCTION

The Cheirodontinae inhabit most neotropical aquatic habitats, its distribution includes large river systems of the Neotropics, comprising 45 regions of the 54 delimited by Abell et al. (2008) from Costa Rica to southern Chile. It is also one of the most widely distributed subfamilies of Characidae, with species found from Costa Rica [*Pseudocheirodon terrabae* Bussing, 1967, *Odontostilbe dialeptura* (Fink & Weitzman, 1974)] to the Cis-Andean rivers of northern Patagonia, *Cheirodon interruptus* (Jenyns, 1842) and Trans-Andean regions in southern Chile, *Cheirodon australe* Eigenmann, 1928 (Campos, 1982; Malabarba, 1998, 2003; Mariguela et al., 2008).

Cheirodontinae is a clade of freshwater fish belonging to the family Characidae, composed of 66 valid species (excluding *Macropsobrycon xinguensis* Géry, 1973) in 16 genera: *Acinocheirodon* Malabarba & Weitzman, 1999, *Aphyocheirodon* Eigenmann, 1915, *Cheirodon* Girard, 1855, *Cheirodontops* Schultz, 1944, *Compsura* Eigenmann, 1915, *Ctenocheirodon* Malabarba & Jerep, 2012, *Heterocheirodon* Malabarba, 1998, *Kolpotocheirodon* Malabarba & Weitzman, 2000, *Macropsobrycon* Eigenmann, 1915, *Nanocheirodon* Malabarba, 1998, *Odontostilbe* Cope, 1870, *Prodontocharax* Eigenmann & Pearson, 1924, *Protocheirodon* Vari, 2016, *Pseudocheirodon* Meek & Hildebrand, 1916, *Saccoderma* Schultz, 1944, *Serrapinnus* Malabarba, 1998, and a fossil genus

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Disclosure statement

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FIGURES

Figure 1: Hypothesis of phylogenetic relationships of Cheirodontinae, modified from (a) Malabarba (1998), morphological data; (b) Mariguela et al. 2013, multilocus molecular data; (c) Mirande et al. (2019), total evidence data; (d) Terán et al. (2020), total evidence data; and (e) Melo et al. (2022), genomic data "UCEs". In (a), Kolpotocheirodon substitutes the 'New gen. & sp. A', Acinocheirodon substitutes the 'New gen. & sp. B', and Ctenocheirodon substitutes the 'New gen. & sp. C' of Malabarba (1998). In (b), the first tribe is Pseudocheirodontini (Mariguela et al., 2013).

Figure 2: Summary of all analyzes realized with Matrix-A and Matrix-B.

Figure 3. Phylogenetic reconstructions using concatenated molecular datasets 1, with Bayesian inference (BI). Posterior probability support values are presented. Clade A: Amblystilbini; Clade B: Pseudocheirodontini; Clade C: Prodontocharacini; Clade D: Cheirodontini; Clade E: Holoshesthini; Clade F: Macropsobryconini; Clade G: Compsurini.

Figure 4. Phylogenetic reconstructions using concatenated molecular datasets with Bayesian inference (BI). Posterior probability support values are presented. Clade A: Amblystilbini; Clade B: Pseudocheirodontini; Clade C: Prodontocharacini; Clade D: Cheirodontini; Clade E: Holoshesthini; Clade F: Macropsobryconini; Clade G: Compsurini.

Figure 5. Phylogenetic reconstructions using concatenated molecular datasets with Species Trees (ST) analysis, Posterior probability support values are presented. Clade A: Amblystilbini; Clade B: Pseudocheirodontini; Clade C: Prodontocharacini; Clade D: Cheirodontini; Clade E: Holoshesthini; Clade F: Macropsobryconini; Clade G: Compsurini.

Figure 6. Phylogenetic reconstructions using Maximum Likelihood (ML) analyzes ran in RAxML v2.0.1 with molecular data. Bootstrap support values are presented. Clade A: Amblystilbini; Clade B: Pseudocheirodontini; Clade C: Prodontocharacini; Clade D: Cheirodontini; Clade E: Holoshesthini; Clade F: Macropsobryconini; Clade G: Compsurini.

Figure 7. Phylogenetic reconstructions using morphological matrix with Maximum Parsimony analysis (MP), GC support values are presented.

Figure 8. Phylogenetic reconstructions using concatenated molecular datasets with Maximum Parsimony analysis (MP) with weighting schemes, Weight by genes (BLK), GC support values are presented. Clade A: Amblystilbini; Clade B: Pseudocheirodontini; Clade C: Prodontocharacini; Clade D: Cheirodontini; Clade E: Holoshesthini; Clade F: Macropsobryconini; Clade G: Compsurini.

Figure 9. Phylogenetic reconstructions using concatenated molecular datasets with Maximum Parsimony analysis (MP) with weighting schemes, Weight by 1st, 2nd, 3rd position (POS), GC support values are presented. Clade A: Amblystilbini; Clade B: Pseudocheirodontini; Clade C: Prodontocharacini; Clade D: Cheirodontini; Clade E: Holoshesthini; Clade F: Macropsobryconini; Clade G: Compsurini.

Figure 10. Phylogenetic reconstructions using concatenated molecular datasets with Maximum Parsimony analysis (MP) with weighting schemes, Weight individual site (SEP), GC support values are presented. Clade A: Amblystilbini; Clade B: Pseudocheirodontini; Clade C: Prodontocharacini; Clade D: Cheirodontini; Clade E: Holoshesthini; Clade F: Macropsobryconini; Clade G: Compsurini.

Figure 11. Phylogenetic reconstructions using concatenated molecular datasets with Maximum Parsimony analysis (MP) with weighting schemes, Equal weights (EW), GC

support values are presented. Clade A: Amblystilbini; Clade B: Pseudocheirodontini; Clade C: Prodontocharacini; Clade D: Cheirodontini; Clade E: Holoshesthini; Clade F: Macropsobryconini; Clade G: Compsurini.

Figure 12. Phylogenetic reconstructions using morphological-molecular matrix with Maximum Parsimony analysis (MPTE) with weighting schemes, final hypothesis is the strict consensus of 12 trees of 6104 steps (Fit 1152.17626; CI = 4.059; RI = 1.191). GC support value is presented. Clade A: Amblystilbini; Clade B: Pseudocheirodontini; Clade C: Prodontocharacini; Clade D: Cheirodontini; Clade E: Holoshesthini; Clade F: Macropsobryconini; Clade G: Compsurini.

Figure 13. The distance between the trees obtained was calculated by TNT, using the normalized Robinson-Foulds distance metric (rf; 0: identical topologies, 1: maximum incongruity).

TABLE

Table 1: Genes partitioned by codon position and the best nucleotide substitution model and partition scheme obtained by PartitionFinder using AICc criteria for each of the analyses; and best model obtained by ModelTest using AIC.

SUPPLEMENTARY INFORMATION

Figure S1 Concatenated species tree (ML: criterion POS) with values of Gene-support frequency. Labels above branches = node number (Nd). Labels below branches = frequency of gene trees in agreement with reference tree (Yellow + green in in the graphical matrix) | frequency of gene trees that present the node (Green in the graphical matrix; Gene-support frequency).

Figure S2. Graphical matrix of the Gene-support frequency for the Species tree (concatenation-based tree, ML: criterion POS). Groups supported by the gene trees (green box); groups supported by the gene trees, but with some of the descendants missing in the gene tree (yellow box); groups not supported by the gene tree (red box).

Figure S3. Consensus tree, of the trees with the most parsimonious hypothesis using IW, with enumeration at the nodes.

Figure S4. Consensus tree, of the trees with the most parsimonious hypothesis using EW, with enumeration at the nodes.

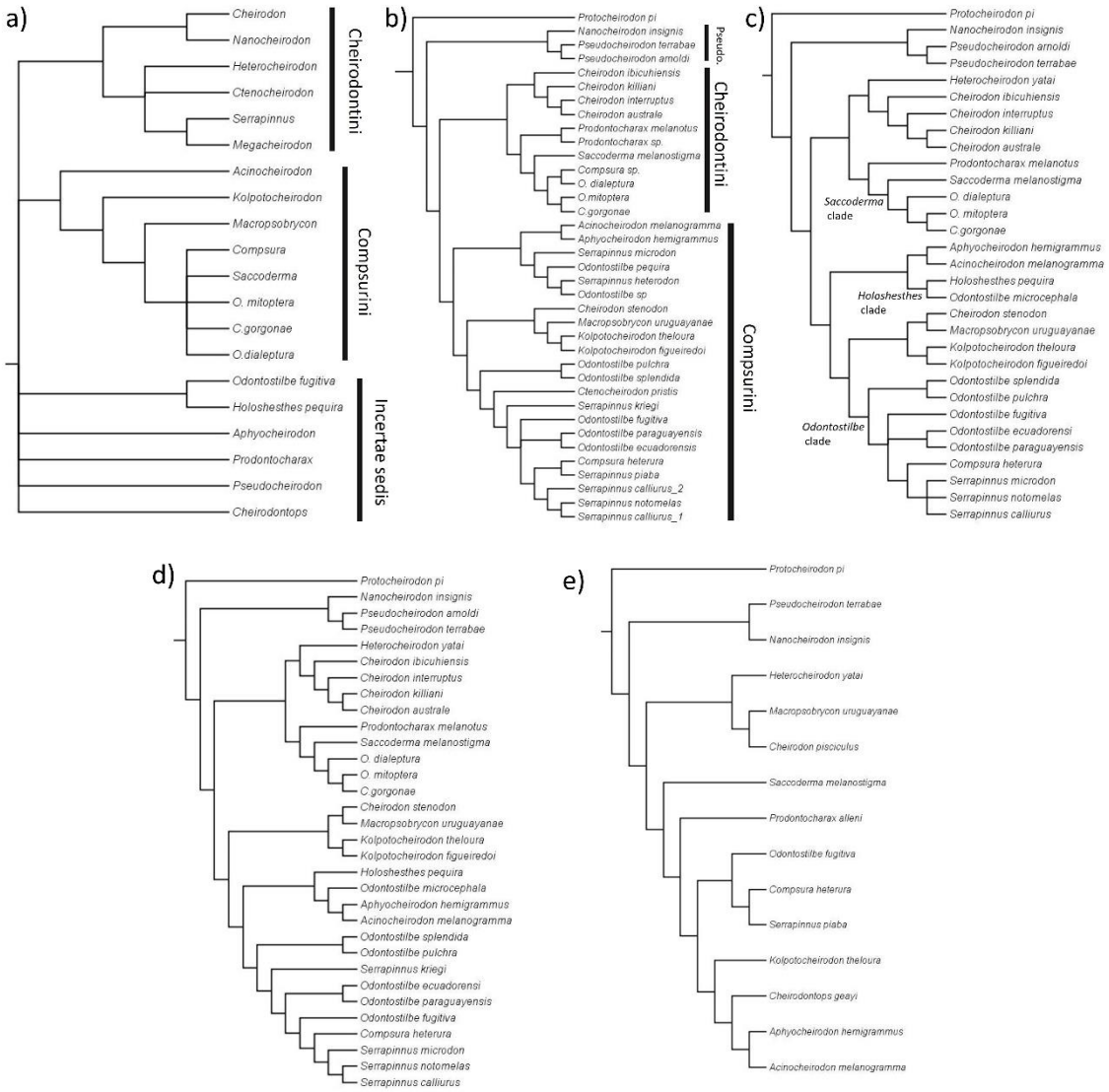
Table S1: List of primers used and optimized PCR conditions for all markers (* Primers that compound the cocktail FishF1t1; ** Primers that compound the cocktail FishR1t1)

Table S2: MATRIX-1: Sequenced species code and Information about GenBank accession numbers

Table: S3: MATRIX-2: Sequenced species code and Information about GenBank accession numbers

Table S4: Morphological characters

Table S5: The morphological matrix



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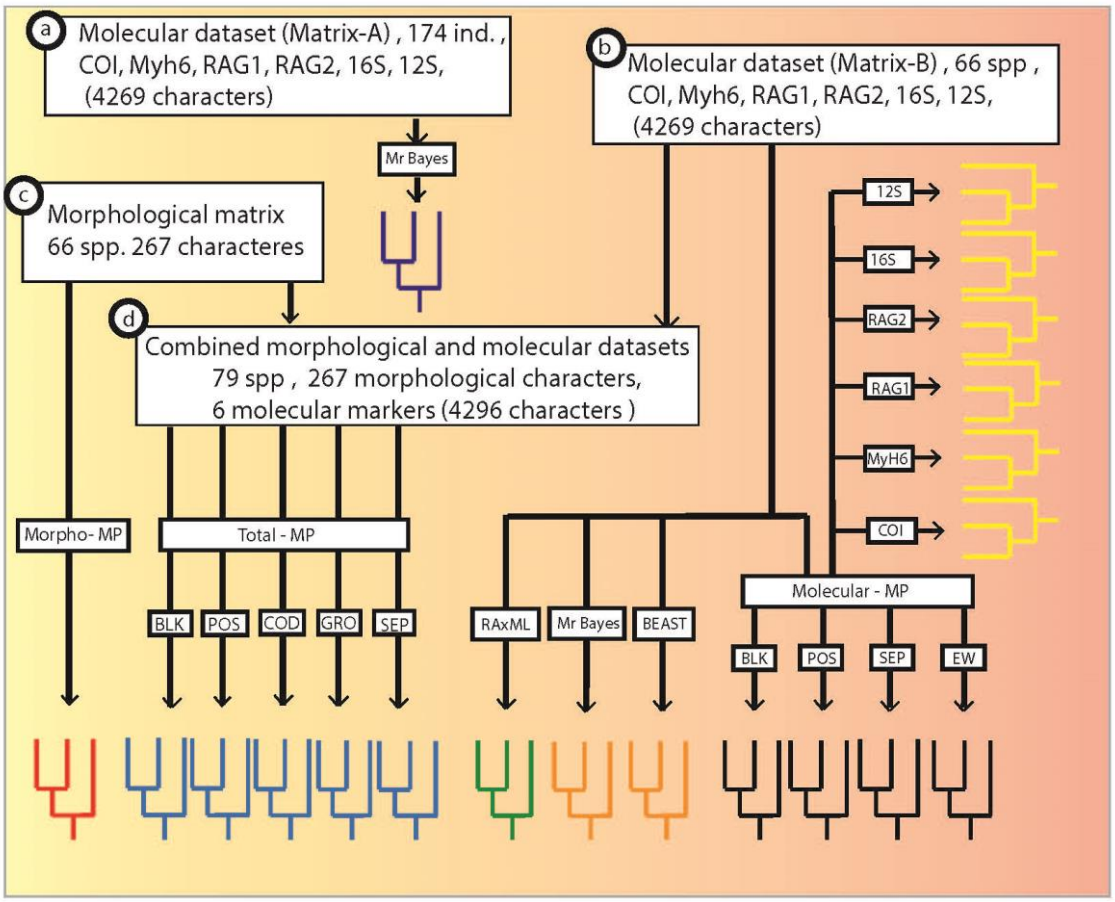

Figure 2: Summary of all analyzes realized with Matrix-A and Matrix-B.

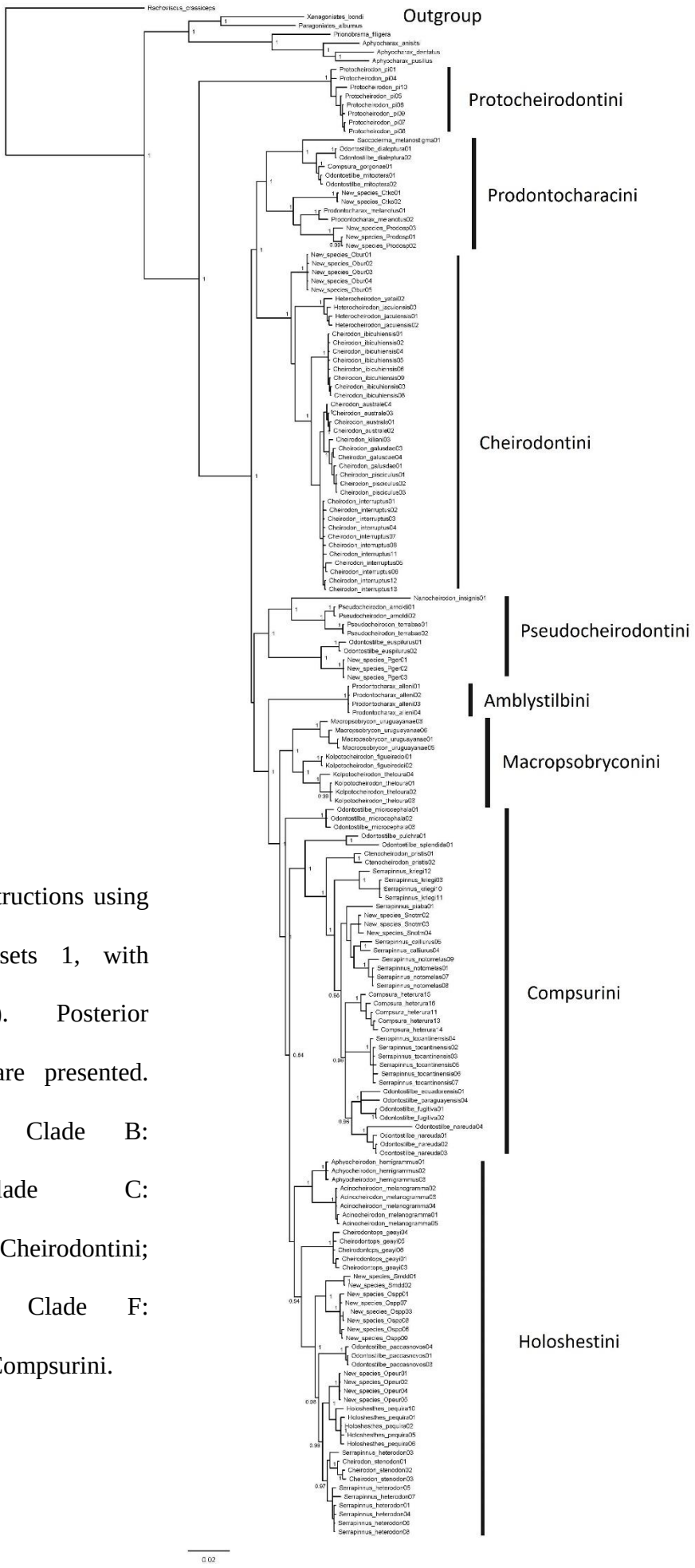

Figure 3. Phylogenetic reconstructions using concatenated molecular datasets 1, with Bayesian inference (BI). Posterior probabilities support value are presented. Clade A: Amblystilbini; Clade B: Pseudocheirodontini; Clade C: Prodontocharacini; Clade D: Cheirodontini; Clade E: Holoshesthini; Clade F: Macropsobryconini; Clade G: Compsurini.

CHAPTER 3

Reassessment of the monophyly of Cheirodontinae lineages with total-evidence Bayesian techniques launches new hypotheses about fish diversification processes in the Neotropical region

Reassessment of the monophyly of Cheirodontinae lineages with total-evidence Bayesian techniques launches new hypotheses about fish diversification processes in the Neotropical region

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ABSTRACT

We present a novel approach to test the monophyly of eight main clades of the Cheirodontinae within Characidae obtained through total evidence analysis based on parsimony. We performed a total-evidence Bayesian analysis in Beast2 including all major Cheirodontinae genera, using the fossilized birth-death (FBD) model and incorporating 267 morphological characters, nuclear and mitochondrial gene sequences, and stratigraphic occurrence data. Our results place the origin of Cheirodontinae at 28.43 ma during the Oligocene. At 22.18 ma in the Miocene, occurred the origin of clade Protocheirodontini, later the clades originated: Pseudocheirodontini (12.65 ma), Prodontocharacini (12.56 ma), Cheirodontini (7.26 ma), Holoshesthini (8.76 ma), Macropsobryconini (7.94 ma) and Compsurini (10.05 ma). Ancestral area estimation performed with BioGeoBEARS using RASP 3.2 best supports a primarily Amazonian paleobiogeographic center for the Cheirodontinae with multiple dispersal events to other basins and multiple vicariance event because of Andes formation to north and south of South American continent. Insemination adaptations have evolved numerous times within Characidae. Some of the most diverse insemination adaptations are found within the subfamily Cheirodontinae. Ancestral state estimation with MK model using make.simmap of phytools in R supported the convergence in the appearance of insemination six times in Cheirodontinae in clades Pseudocheirodontini(2), Prodontocharacini, Holoshesthini, Macropsobryconini and Compsurini, a relationship not found in prior cladistics studies. Results support the hypothesis of eight clades within Cheirodontinae obtained previously by parsimony analysis.

Keywords: Ancestral character, Ancestral area, Biogeography, Macroevolution.

INTRODUCTION

The evolutionary origin and maintenance of high Neotropical biodiversity have been a controversial topic (Rull, 2008, Rull, 2011), and are not yet fully understood. The successive influences of Paleogene-Neogene and Quaternary events are hypothesized to have driven the differentiation of communities from open biomes and adjacent rainforests (Rull, 2011) and several hypotheses linking mechanisms of diversification to regional geomorphological and climate histories have been proposed (Lundberg et al., 1998; Albert & Reis, 2011; Albert et al., 2020). Events since the fragmentation of western Gondwana in Early/Late Cretaceous, including the increase of the latitudinal temperature gradient in South America caused by the Andean orogeny in the Paleogene (Granot & Dyment, 2015; Lundberg, 1993), the paleogeographic transformation of the Caribbean-draining Proto–Orinoco–Amazonas (POA) basin to the modern Amazon and Orinoco basins (Albert et al., 2018; Hoorn et al., 2010), marine transgressions during the Miocene, as well as global climate oscillations (Abreu et al., 2019; Bloom & Lovejoy, 2011; Thomaz & Knowles, 2018; Thomaz et al., 2015), and the uplift of the Central Brazilian Plateau in the Miocene-Pliocene transition, were some events that led to paleogeographical reorganizations that may have directed diversification. These processes, together with the Quaternary climatic fluctuations, changed the landscape (Vuilleumier, 1971), biodiversity through time (Lundberg, 1993; Lundberg et al., 1998; Hoorn et al., 2010; Albert & Reis, 2011; Albert, Val, et al., 2018; Tagliacollo et al., 2015; Albert et al., 2020; Fontenelle et al., 2021), and created complex scenarios that may have fostered both diversification and extinction events. Such complexity has been hypothesized in many phylogeographic and phylogenetic studies where Neogene and Pleistocene diversifications have been recovered (Cardoso et al. 2021, Melo et al. 2021, Frable et al. 2022). In addition, climatic fluctuations remain one of the main mechanisms

to promote fluctuation of population distributions through time in South American species (Thomaz & Knowles, 2018; Thomaz et al., 2015).

Currently, Considerable uncertainty remains about the precise links between the region's geomorphological history and the evolutionary origin, geographical distribution and diversification pattern of modern Neotropical fish fauna. Understanding when and/or how members of a clade have dispersed to some places and not to others is essential to recognize large-scale patterns of clade distribution (Cardoso et al. 2021). For freshwater fishes, distribution patterns and dispersal dynamics must be interpreted in the context of the geological and geographic histories of river basins (Reis et al. 2016), because the ability of strictly freshwater fish to move between drainages, is limited by the hydrological connectivity (Cardoso et al 2021).

Among Neotropical freshwater fishes, the subfamily Cheirodontinae is the fourth most species-rich subfamilies among Characidae and has members in all Neotropical basins. Thus, Cheirodontinae is an excellent model to study the effects of landscape evolution on fish lineage diversification, and comprehend the events that have given rise to the current diversity of fish species in the Neotropical region. Understanding the causes of this species richness requires detailed species-level knowledge of geographic distribution, ecologically relevant traits and phylogenetic relationships. Over the last 25 years, few investigations of phylogenetic relationships of Cheirodontinae have been published, but there have been important discoveries (new species and genera) and changes in their classification have been proposed (Malabarba, 1998, Mariguela, 2013, Mirande, 2019; Melo, 2022; Chuctaya, chapter 2). However, there are some conflicting phylogenetic relationships within the subfamily, such as in lineages with fast diversification (*Odontostilbe*, *Serrapinnus*), or due to the under-representation of species coming from particular river basins (*Nanocheirodon*, *Saccoderma*, *Compsura*, *Amblystilbe*). These

remaining challenges limit the investigation of important questions regarding the evolution of these Neotropical freshwater fishes.

Comprehensive documentation of extant and fossil species is required to understand the biogeographical history of the region, and these integrative approaches combining morphological and molecular evidence are becoming increasingly common practice (Ronquis et al. 2012; Slater & Harmon, 2013; Arcila & Tyler, 2015; Arcila et al. 2017). The fossils provide an extraordinary window for understanding past evolutionary events because they can offer information about the direction of character evolution, the timing of divergence, diversification rates and biogeography (Arcila et al. 2017). Accurate calibration of molecular clocks to estimate divergence times relies critically on the interpretation of paleontological information, particularly on the inferred ages of fossils and their phylogenetic placement, where fossil taxa are typically used in morphological phylogenies as terminals and in molecular phylogenetic as constraints or priors for minimum or maximum ages on the divergence times of internal nodes (Arcila & Tyler, 2015).

The objective of this work is to used the subfamily Cheirodontinae as a model group to determine from where and when the early lineages colonized the different basins of the Neotropics, and how they diversified relatively to the intense orogenic changes observed in the Neotropics. We address these questions by inferring a time-calibrated phylogeny using extant and fossil species, generated from morphological and molecular data, with an almost complete representation of all species that make up the subfamily Cheirodontinae that are distributed throughout this region. Using our chronogram, we estimated the ancestral distribution ranges, which allows us to propose an integrated view of the diversification history of the Cheirodontinae lineage in the Neotropical region.

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FIGURES

Figure 1: MBTE-BI analysis showing interrelationships among Cheirodontinae. Relationships among these subclades are similar to previous molecular studies, except for the position of the clade A, which is proposed here as the sister group of clades E, F, and G. Nodes with PP = posterior probability. Red = species with only morphological character. Blue = species with only molecular character. Clade A: Amblystilbini; Clade B: Pseudocheirodontini; Clade C: Prodontocharacini; Clade D: Cheirodontini; Clade E: Holoshesthini; Clade F: Macropsobryconini; Clade G: Compsurini.

Figure 2. Time-calibrated phylogeny of Cheirodontinae species and outgroup, Total-Evidence with Fossilized Birth Death Model Dating (FBD). Time bar as Million Years (Ma). Clade A: Amblystilbini; Clade B: Pseudocheirodontini; Clade C: Prodontocharacini; Clade D: Cheirodontini; Clade E: Holoshesthini; Clade F: Macropsobryconini; Clade G: Compsurini.

Figure 3: Biogeographic reconstruction, using the DIVA-like model. Map with distribution areas: (A) Amazonas; (B) La Plata basin; (C) Atlantic coastal drainages; (D) South Pacific drainages; (E) Orinoco-Guianas; and (F) Trans-Andean. Biogeographic hypotheses for dispersal routes in Group 1 and Group (2) from Miocene to Quaternary based on the results from RASP using BioGeoBEARS.

Figure 4. (a) Tempo and mode of macroevolutionary diversification in Cheirodontinae. A lineage through-time (LTT) curve showing species accumulation over time (dashed line red) against that predicted under Brownian motion (solid line black). Confidence intervals are shown at 95% (dashed line black). The phylogeny of Cheirodontinae is shown in the background of the LTT plot. (b) Estimated speciation (λ) and extinction (μ) rates of the Cheirodontinae are presented in density plots. Images of Cheirodontinae are from top to bottom: (c) *Cheirodon ibicuhiensis*, (b) *Macropsobrycon uruguayanae*, and (c) *Heterocheirodon yatai*, photo by Luiz Malabarba.

Figure 5. Best model of variable diversification in time and between clades de Cheirodontinae: (1) Clade (Amblystilbini, Pseudocheirodontini, Holoshesthini, Macropsobryconini, Compsurini) (Fig. a, b, c); (2) Clade (Prodontocharacini, Cheirodontini) (Fig. d, e. f); (a,d) Fitted net diversification rate, (b,e) Fitted speciation rate; and (c,f) Fitted extinction rate.

Figure 6: (a) Diversification rate in Cheirodontinae, (b) Regression analysis of body size vs Tip Rate Diversification (DR)

Figure 7. (a) Maximum likelihood phylogenetic reconstruction of ancestral body sizes (ln-transformed) using the Ornstein-Uhlenbeck (OU) model, with the interspecific range shown in the coloured bar. (b) Fig: Disparity in body size among species of Cheirodontinae, Mean subclade disparity through-time (DTT) for body size (solid line) compared with median subclade DTT under Brownian motion (dashed line). The grey area represents the 95% confidence interval of DTT range based on simulations of body size. Median subclade disparity was calculated based on 999 simulations of phenotypic evolution on the Cheirodontinae phylogeny.

Figure 8. Projection of the Cheirodontinae phylogeny onto a morphospace of body size (ln-transformed) as a function of time (in million years elapsed since the root). Species size limits are observed, with *Odontostilbe avanhandava* being the largest and *Kolpotocheirodon theloura* being the smallest.

Figure 9. Maximum likelihood phylogenetic reconstruction of ancestral insemination using the Equal rates (ER) model, with the interspecific range shown in the coloured bar.

TABLES

Table 1: Genes partitioned by codon position and the best nucleotide substitution model and partition scheme obtained by PartitionFinder using AICc criteria for each of the analyses; and best model obtained by ModelTest using AIC.

Table 2. Models and AICc_wt scores for three biogeographic models

Table 3 Rates of species accumulation during Cheirodontinae diversification history based on two evolutionary models

Table 4. Models of variable diversification in time and between clades de Cheirodontinae based on four evolutionary models

Table 5. Rates and modes of evolutionary diversification in Cheirodontinae body size based on comparisons of three evolutionary models. Fitted models are Brownian-motion

(BM), Ornstein-Uhlenbeck (OU), and Early-burst (EB) Best fit models based on the (delta) Akaike Information Criteria (AIC)

Table 6 Models of variable diversification in time and between clades de Cheirodontinae based on six evolutionary models.

Table 7 Rates and models of evolutionary diversification in Cheirodontinae insemination based on comparisons of two evolutionary models. Fitted models are Equal rates (ER) and All rates different (ARD). Best fit models based on the (delta) Akaike Information Criteria (AIC)

SUPPLEMENTARY MATERIAL

Table S1: Distribution areas of Cheirodontinae (presence=1; absence=0)

Table S2: Information on size and inseminating or non-inseminating character in Cheirodontinae: SL= standard length.

Table S3. Information on speciation events by nodes, result of the analysis of DIVA-LIKE in RASP-4 Software.

CHAPTER 4

A new species of *Odontostilbe* Cope (Characiformes: Cheirodontinae) from rio Madeira basin diagnosed based on morphological e molecular data

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A new species of *Odontostilbe* Cope (Characiformes: Cheirodontinae) from rio Madeira basin diagnosed based on morphological e molecular data

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Running title: New *Odontostilbe* from rio Madeira

Abstract

A new species of *Odontostilbe* is described from the rio Jaciparaná, rio Madeira basin, Rondônia, Brazil. *Odontostilbe pacaasnovos*, differs from all its congeners, except *O. pequira*, by the colour pattern. Additionally, it differs from its congeners by the terminal mouth, number of cusps in the teeth of the premaxilla (5–7), number of branched rays in the anal fin (19–22), by the shape of dentary teeth with 5–7 cusps being central cusp larger and longer than laterals cusps, and by the number of lamellae of the olfactory rosette (17–18 in male and 14 in female). Morphological and molecular comparisons corroborate the distinctiveness between *Odontostilbe pacaasnovos* and its congeners, being recognized as a new species.

KEYWORDS. Amazon, COI, DNA barcode, genetic distance, taxonomy

1 INTRODUCTION

The rio Madeira is one of the main tributaries of the Amazon river and is known for its high diversity with 1406 species, representing 58 % of the valid species of the Amazon basin (Jézéquel *et al*., 2020). The family Characidae, with 635 valid species from rio Madeira, represents 49% of the species registered for the Amazon basin (Dagosta & de Pinna, 2019); 13 species of Cheirodontinae were recorded from rio Madeira (Lima *et al*., 2013).

Fricke *et al*. (2020) list 65 species valid for Cheirodontinae. Malabarba (1998), Jerep & Malabarba (2011) and Jerep *et al.* (2017), however, considered '*Cheirodon*' *luelingi* Géry and '*Cheirodon*' *ortegai* Vari & Géry from the Ucayali basin, and '*Macropsobrycon*' *xinguensis* Géry from the Xingu basin, as species that do not have diagnostic characters of Cheirodontinae, *Cheirodon*, or *Macropsobrycon* and should belong to other characid groups. Additionally, Bührnheim & Malabarba (2006) consider *Odontostilbe roloffi* Géry a junior synonym of *O. euspilurus* (Fowler). Following these observations, currently Cheirodontinae would sum 61 valid species. The subfamily is diagnosed by four characters: presence of a large, nearly triangular pseudotympanum between the first and second pleural ribs; absence of humeral spot; teeth pedunculated, expanded and compressed distally; and one series of teeth in the premaxilla (Malabarba, 1998; Jerep *et al*., 2017).

Odontostilbe Cope is the most diverse genus within Cheirodontinae, with 16 valid species (Chuctaya *et al.*, 2018). Hypothesis of phylogenetic relationships with molecular markers (Mariguela *et al*., 2013) and the combination of molecular and morphological markers (Mirande, 2018) have hypothesized the genus as non-monophyletic based on a few representatives, but no new morphological diagnosis has been presented for *Odontostilbe*.

In recent years, species delimitation has included barcode and other DNA information along with morphological characters, giving an integrative approach to the description of species. This approach has speeded up species discovery, particularly in morphologically challenging and hyper-diverse groups (Britz *et al*., 2020) - one of the groups that presents these characteristics are the Cheirodontinae.

The aim of the present contribution is to describe a new species of *Odontostilbe* based on the current diagnosis of the genus (Malabarba, 1998) and recently used in the description of new species within the genus (Bührnheim & Malabarba, 2006, 2007; Chuctaya *et al*., 2018). The new species presents a pattern of coloration similar to *Odontostilbe pequira* (Steindachner) from the La Plata river basin. The distinctiveness between the two species is demonstrated with morphological, morphometric and molecular evidences.

2 MATERIAL AND METHODS

The fish sampling was authorized by João Ribeiro (ICMBio) and collection permit was granted by the Sistema de Autorizacão e Informacão em Biodiversidade (SISBIO 65866-1). Captured individuals were anaesthetized and sacrificed by immersion in eugenol, fixed in 10% formalin solution and later preserved in 70% ethanol. The fish were deposited in the ichthyological collection of the Universidade Federal do Rio Grande do Sul (UFRGS). No surgical or experimental procedures were performed with live fish.

2.1 Morphological analyses

Counts and measurements follow Fink & Weitzman (1974) with modification detailed in Chuctaya *et al*. (2018). All measurements were taken with a digital calliper to the nearest

0.1 mm. Standard length (SL) is expressed in millimetres (mm), all other measurements are expressed as percentages of SL, except head measurements, which are expressed as percentages of head length (HL). Measurements, whenever possible, were taken from the left side of specimens. Precaudal, caudal and total vertebrae count include the four vertebrae of the Weberian apparatus, and the terminal "half centrum" as outlined by Malabarba & Weitzman (1999). In the counting of the rays of anal fin, the last two rays are counted as a single element by being on the same pterygiophore. Specimens were cleared and stained (c&s) according to Taylor & Van Dyke (1985), and were used for counting vertebrae, dorsal and anal fin unbranched rays, dorsal and ventral procurrent rays, teeth, gill rakers, denticulation of gill rakers, supraneurals and dorsal and anal fin proximal radials. The count of lamellae of olfactory organ was performed with the head immersed in 70° alcohol; the tissue that joins the two nostrils (anterior and posterior nostril), the antorbital and nasal bones were removed to have a complete view of the lamellae. Each count is presented with the frequency registered in parentheses; holotype counts marked with asterisk. Photos of dentitions and lamellae of olfactory organ were taken with a Nikon AZ100M camera attached to a stereomicroscope. Institutional abbreviation follows Sabaj (2019).

2.2 Molecular analyses

2.2.1 DNA extraction, amplification and sequencing

Voucher of the specimens used in molecular analysis are deposited at UFRGS fish Collection. DNA extraction was performed using 20 mg of tissue following the CTAB method described by from Doyle & Doyle (1987). Partial sequences of COI gene were obtained from 11 individual of *Odontostilbe* using the primer designed by Melo *et al*. (2011): COI L6252-Asn (5'- AAG GCG GGG AAA GCC CCG GCA G -3') and H7271-COXI (5'- TCC TAT GTA GCC GAA TGG TTC TTT T -3'). Amplifications were

performed in a solution with a total volume of 20µl with 13.4µl ultrapure H_2O, 2.0µl DNA buffer (10x), 2.0µl dNTP (8mM), 1.0 µl $MgCl_2$ (10mM), 0.2 µl primer L6252-Asn and 0.2 µl primer H7271-COXI, 0.2 µl Taq polymerase enzyme and 1.0 µl genomic DNA (10-50 ng). The thermocycling program from the polymerase chain reaction (PCR) was as follows: first denaturation step of 95°C for 4 minutes, followed by 35 cycles of 95°C for 30 seconds, 50°C annealing temperature for 45 seconds, 72°C for 45 seconds and a final extension step of 72°C for 10 minutes (following Melo *et al*.. 2011, with modifications). The PCR product were identified in a 2% agarose gel, and the amplified segment were purified using ExoSap-IT® enzyme, following instructions of the manufacturer. The PCR product was sent to ACTgene (Brazil) for sequencing. Four sequences of *Odontostilbe pequira* were used from the GENBANK platform with access code: KU288885.1, KU288858.1, KU288856.1, and KU288855.1 (Diaz *et al*., 2016).

2.2.2 Edition, alignment and phylogeny inference

Mitochondrial gene cytochrome c oxidase subunit 1 – COI (539 bp), was chosen because it is the universal barcode gene and widely available for comparative data. A consensus sequence was generated from chromatograms of forward and reverse sequences using the software Geneious 8.1.4. (Drummond *et al*., 2010). The alignment of sequences of COI was made using MUSCLE algorithm, implemented in the software Geneious under default parameters. Alignments of coding region were visually inspected to ensure correct reading frame (i.e., absence of stop codons).

Phylogenetic analysis was inferred the Neighbor-Joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Paired

genetic distances between sequences of *Odontostilbe* were calculated using the Kimura 2-parameter (K2P) model (Kimura, 1980) in MEGAX (Kumar *et al*., 2018).

For the construction of haplotype networks, the Median-joining network method (Bandelt *et al*., 1999) implemented in the NETWORK program (https://www.fluxus-engineering.com/sharenet.htm) was used. The DnaSP program was previously used for the creation of the extension file (.rdf) that is used to calculate and graph the haplotype network in the NETWORK program. The haplotype network graphic is composed of circles that represent the haplotypes (size proportional to the number of individuals), number of mutations is represented by number in the branches that connect the haplotypes. Additionally, information of the medians vectors (mv) are presented. They are sequences of hypothetical haplotypes.

DNA sequences were obtained from tissues of following samples: *Odontostilbe fugitiva* (UFRGS 28507, UFRGS 28508); *Odontostilbe pequira* (UFRGS 13507, UFRGS 19414, UFRGS 16647, UFRGS 28506), *Odontostilbe nareuda* Bührnheim & Malabarba (UFRGS 21687, UFRGS 21731, UFRGS 21785), *Odontostilbe paraguayensis* Eigenmann & Kennedy (UFRGS 13584), *Odontostilbe microcephala* Eigenmann (CI-FML 7335), and *Odontostilbe pacaasnovos.* (UFRGS 26296). These sequences were deposited in GENBANK with the codes MT823243- MT823255.

3 RESULTS

3.1 *Odontostilbe pacaasnovos*, new species

urn:lsid:zoobank.org:act:D23FEE71-107C-43D5-8C27-7EBFD07BC712

(Figures 1–4 and Table 1)

3.1.1 Holotype.

UFRGS 28509, 27.8 mm SL, male, Brazil, Rondônia, Campo Novo de Rondônia, Madeira basin, upper rio Jaciparaná, Parque Nacional Pacaás Novos, 10°31'32.0"S 63°58'22.0"W, 18 Aug 2018, W.M. Ohara & D.B. Hungria.

3.1.2 Paratypes.

UFRGS 26291, 33, 25.1–31.5 mm SL, 14 males, 19 females, (plus 6 c&s, 28.0–30.7 mm SL, 5 males, 1 female), same data as holotype. **MZUSP 125679**, 20 (25.6–30.2 mm SL), same data as holotype. **INPA 59406**, 20 (25.5–29.0 mm SL), same data as holotype. **MNRJ 51769**, 20 (24.8–29.5 mm SL), same data as holotype.

3.1.3 Non-types specimens.

UFRO-ICT 23431, 3, 24.0–26.1 mm SL, same data of locality as holotype, 13 Dec 2013, D. Hungria, A. Cella-Ribeiro & J. Ribeiro.

3.1.4 Diagnosis.

Odontostilbe pacaasnovos differs from all species of the genus, except from *Odontostilbe pequira* by the presence of a conspicuous dark blotch on the distal part of the dorsal fin. *Odontostilbe pacaasnovos* differs of *O. pequira* by anterior dentary teeth with 5–7 cusps, being central cusp larger and longer than laterals cusps (*vs*. dentary with four anterior teeth with three large and equally longer compressed cusps and 2–3 lateral small cusps), by the number of lamellae of olfactory organ, with 17–18 in male and 14-15 in female (*vs*. 36–40 in male and 19–25 in female, in *O. pequira*). Additionally, *Odontostilbe pacaasnovos* can be easily distinguished by the terminal mouth (*vs*. subterminal mouth in *O. avanhandava Chuctaya, Bührnheim & Malabarba, O. dierythrura* Fowler, *O. euspilurus* (Fowler) and *O. microcephala*); by the presence of hook on first to 12th

branched anal-fin rays in males (vs. hook on first to 16th in *O. ecuadorensis* and hooks on first to 22nd in *O. pulchra* (Gill); by the presence of 5–7 [mostly 5] cusps in premaxillary teeth (*vs.* 8–10 cusps in *O. parecis* Bührnheim & Malabarba and *O. pao* Bührnheim & Malabarba; 8–11 cusps in *O. splendida* Bührnheim & Malabarba, and 9-11 in *O. fugitiva*); by the number of branched rays of anal-fin, 18–22 (*vs.* 24–26 in *O. nareuda*); by the presence of 4 or 5 separate supraneurals (*vs.* 2nd - 4th supraneurals fused in *O. paraguayensis* Eigenmann & Kennedy); and *by the absence of mesopterygoid teeth (vs. present in O. weitzmani Chuctaya, Bührnheim & Malabarba).*

3.1.5 Description.

For overall appearance see Fig. 1. Morphometric data of holotype and paratypes are provided in Table 1. Body laterally compressed, greatest body depth at vertical through dorsal-fin origin. Dorsal profile of head slightly convex from tip of snout to posterior limit of supraoccipital, slightly convex to straight from that point to dorsal-fin origin, Dorsal-fin base straight and posteroventrally slanted, and slightly concave to straight from this point to anteriormost dorsal procurrent caudal-fin rays. Ventral body profile convex from tip of lower jaw to pelvic-fin origin, straight or slightly convex from this point to anal-fin origin, straight and posterodorsally slanted along anal-fin base. Ventral profile of caudal peduncle slightly concave.

Mouth terminal, anterior tip of premaxilla horizontally aligned with centre of eye. Premaxilla with one row of teeth, with five teeth (one specimen with seven teeth on right side). Maxilla with three (5) or four (1) teeth, usually with 5–7 cusps. Posterior tip of maxilla approximately at vertical through anterior margin of eye. Dentary teeth in single row; teeth near mandibular symphysis with 5–7 cusps, being central cusp larger and longer than other cusps, gradually decreasing in size, followed by small tricuspid and

conical teeth (Fig. 2). Olfactory rosette slightly oval with 14(2) lamellae in females, 17(1) and 18(1) lamellae in males around of central median raphe (Fig. 3).

Dorsal-fin rays ii, nine* (40). Dorsal-fin origin at posterior half of body, and slightly anterior to vertical through anal-fin origin. Pterygiophores with ten proximal-middle radials with serially associated distal radials (6c&s). First to 7th pterygiophores with lateral projection. First proximal-middle radial with anterior edge projecting in direction of supraneurals. Pectoral-fin rays i, 10(11), 11(22)*, or 12(1). Tip of pectoral fin reaching beyond vertical through pelvic-fin insertion. Pelvic-fin rays i, 7(40)*. Pelvic-fin origin anterior to half of body. Pelvic-fin tip not reaching anal-fin origin. Anal-fin rays iv(1) or v(5) (6c&s), 18(2), 19(7), 20(11)*, 21(5), or 22(1). Anal-fin origin at posterior half of body. First anal-fin pterygiophore situated posterior to hemal spine of first caudal vertebrae, supporting all unbranched anal-fin rays. Adipose fin present. Caudal fin forked with i,9/8,i* principal rays in all specimens (40); dorsal procurrent caudal-fin rays 8(1), 11(2), 12(2), or 13(1), ventral procurrent caudal-fin rays 9(1) or 10(5).

Scales cycloid. Lateral line complete with 34(3), 35(12)*, 36(13), 37(5), or 38 (1) scales. Scale rows between dorsal-fin origin and lateral line five (34). Scale rows between lateral line and pelvic-fin origin four (34)*. Circumpeduncular scales 14(34)*. Total number of vertebrae 32(1), 33(4), or 34(1). Precaudal vertebrae 15(6), caudal vertebrae 17(1), 18(4), or 19(1). Supraneurals 4(1), 5(3), or 6(1), with anteriormost very reduced. First gill arch with 2(1) gill-rakers on hypobranchial, 10(1) on ceratobranchial, 1(1) on cartilage between ceratobranchial and epibranchial, and 6(1) on epibranchial. Branchiostegal rays 4(1).

3.1.6 Colour in alcohol

Overall body colour light yellow to brown (Fig. 1). Black chromatophores densely concentrated on dorsal surface of head, premaxilla, anterior portion of maxilla and

dentary, antorbital, and infraorbital 1 and 2. Infraorbitals 3-6 silvery. Ventral portion of head less pigmented, except by presence of scattered black chromatophores on anterior portion of lower jaw. Scales above midlateral band delineated by black chromatophores. Humeral region with darkened triangular area due to muscular hiatus of pseudotympanum. Narrow, inconspicuous longitudinal dark stripe formed by underlying chromatophores at horizontal septum from region above pseudotympanum to caudal peduncle. Well-defined, black, round caudal-peduncle spot, extending from posterior portion of caudal peduncle to base of middle caudal-fin rays, not extending to tip of fin rays. Dorsal fin with conspicuous black blotch on the half-distal portion, with dark chromatophores densely concentrated mainly over interradial membranes. Pectoral, pelvic and adipose fins hyaline. Anal fin with scattered dark chromatophores mainly in distal region of anterior five branched rays.

3.1.7 Colour in life

Based on pictures taken in the field of specimens collected with the holotype. Head (mostly infraorbitals and opercle) and belly white silvery. Upper margin of iris orange. Body yellowish, with a midlateral greyish silvery stripe from pseudotympanum to caudal peduncle spot, and series of black lines aligned with muscles of anal-fin pterygiophores. Scales above midlateral band delineated by black chromatophores. Dorsal fin dark orange, with black blotch formed by chromatophores densely concentrated on interradial membranes of half-distal portion of dorsal fin. Adipose fin dark orange. Pectoral and pelvic fins mostly hyaline, with scattered orange chromatophores. Anal fin with basal portion of anterior five branched rays dark orange. Caudal peduncle and caudal-fin base with conspicuous round black spot that reaches base of middle caudal-fin rays; proximal portion of caudal-fin lobes dark orange (Fig. 4).

3.1.8 Sexual dimorphism

Biggest male reaching 31.5 mm SL, and female 31.3 mm SL (Table 1). Mature males with small hooks on pelvic- and anal-fins rays. Pelvic fin with small hooks in all branched rays, with two retrorse bony hook per segment mainly in the posterior branch. Tip of bony hook not reaching proximal border of segment where inserted. Anal-fin rays with one pair of small retrorse bony hook per segment symmetrically arranged, present from last unbranched ray to 8^{th} – 12^{th} branched rays, decreasing in number posteriorly. Distal tip of bony hooks not reaching proximal border of segment of lepidotrichia where inserted. Number of the **o**lfactory rosette lamellae in female 14 (2) and males 17(1) or 18(1) (Fig. 3).

3.1.9 Molecular analysis

The sequences from 17 specimens resulted in a matrix with 539 base pairs (pb) for each sequence, from which 428 positions were conserved, and 111 were variable, 85 positions were parsimony informative. The nucleotide frequencies were 29.2% thymine/uracil, 27.1% cytosine, 26.1% adenine, and 17.6% guanine. Genetic distances are presented in Table 2. Phylogenetic relationship was inferred using Neighbor-Joining method (Figure 5).

Intraspecific genetic distance varies between species, *Odontostilbe pacaasnovos* (0.00%); *O. pequira* (0.00%–4.05%); *O. microcephala* (0.00%); *O. paraguayensis* (0.00%); *O. fugitiva* (0.00%), and *O. nareuda* (0.00%–0.37%). Interspecific genetic distance varied between 1.51% (*O. fugitiva* and *O. nareuda*) to 12.07% (*O. pequira* and *O. nareuda*). All sequences of the same species formed groups with high bootstrap support, without any overlap between species. *Odontostilbe pequira* formed two well-supported clades, the Paraná-Paraguay clade and the Uruguay clade. Five new COI barcode records not previously studied were generated and incorporated into the

GENBANK Data System (*O. pacaasnovos*, *O. fugitiva*, *O. microcephala*, *O. nareuda*, and *O. paraguayensis*). Additionally, COI barcode of the population of *Odontostilbe pequira* of the Uruguay basin is incorporated. COI barcode references of these species, will contribute to their molecular identification and their use in studies of phylogenetic relationships.

The haplotype network shows 12 haplotypes (Fig. 6), *Odontostilbe pacaasnovos* with 1 haplotype (H1 from Madeira basin), *O. pequira* presented 6 haplotypes (H2 from Uruguay basin; H3, H4, H5 from Parana basin; H6 and H7 from Paraguay basin); *O. paraguayensis* with 1 haplotype (H9 from Paraguay basin); *O. microcephala* with 1 haplotype (H8 from Parana Basin); *O. fugitiva* with 1 haplotype (H10 from Ucayali basin); and *O. nareuda* with 2 haplotypes (H11 and H12 from Jurua basin). *Odontostilbe pacaasnovos* is nested with *O. pequira*, separated by 23 mutations, and by the following number of mutations from other species of the genus: 76(*O. nareuda*), 77(*O. fugitiva*), 68(*O. paraguayens*is), and 152(*O. microcephala).* The presence of hypothetical haplotypes (9 mv) is also observed.

3.1.10 Geographic distribution

Odontostilbe ***pacaasnovos*** **is** known from the upper rio Jaciparaná, **r**io Madeira drainage, Parque Nacional Pacaás Novos, Campos Novos de Rondônia, Rondônia State**,** Brazil (Fig. 7).

3.1.11 Etymology

This species is named after the Parque Nacional Pacaás Novos. A noun in apposition

3.1.12 Ecological notes

The type-locality of *Odontostilbe* ***pacaasnovos*** is a clear water river 15-25 m wide, 0.5-2 m deep, 156 meters above sea level, with swift current, rocky bottom, and presenting moderate riparian vegetation (Fig. 8). Syntopic species included *Ancistrus* sp., *Astyanax* aff. *bimaculatus*, *Brachychalcinus copei* (Steindachner), *Bryconops* cf. *giacopinii* (Fernández-Yépez), *Ctenobrycon spilurus* (Valenciennes), *Hemiodus unimaculatus* (Bloch 1794), *Jupiaba zonata* (Eigenmann), *Lasiancistrus schomburgkii* (Günther), *Moenkhausia oligolepis* (Günther), *Moenkhausia hasemani* Eigenmann, *Rineloricaria* cf. *phoxocephala* (Eigenmann & Eigenmann), *Roeboides affinis* (Günther) and *Spatuloricaria evansii* (Boulenger 1892).

3.1.13 Conservation status

Odontostilbe pacaasnovos is so far known only from the rio Jaciparaná and its conservation status is uncertain based on the currently available data of its geographic distribution. The new species was captured at the limit of a conservation unit (Parque Nacional do Pacaás Novos), where it is relatively abundant. The new species possibly also occurs within the conservation unit and no imminent threats to the species were detected in the area of occurrence. Consequently, *Odontostilbe pacaasnovos* should be classified as Least Concern (LC) according to the International Union for Conservation of Nature (IUCN) categories and criteria (IUCN Standards and Petitions Subcommittee, 2014).

4 DISCUSSION

The present study represents the first description of species within the Cheirodontinae subfamily that integrates molecular and morphological analysis. The use of molecular analysis provides valuable resources that complement the diagnosis of the species based

on traditional morphological analysis (osteology, external morphology, meristic, and morphometric). Molecular data (COI sequences), however, are still limited for comparisons, being the new species described here compared to five species, corresponding only to 31% of the total species recognized for the genus.

Due to the presence of a black blotch on the dorsal fin, *Odontostilbe pacaasnovos* has an external appearance that resembles that of *O. pequira* from the Paraguay river basin, but it completely lacks the typical three large central cusps arrangement of the dentary teeth of the last species, earlier proposed as diagnostic of the genus *Holoshesthes*. In fact, among compared species, the tree obtained from COI gene indicated a close relationship between *Odontostilbe pacaasnovos* and *O. pequira*. A common biogeographic history between Madeira (mainly species from the sub-basins of the Guaporé and Mamoré rivers) and Paraguay basins have been hypothesized for other groups of freshwater fish, and may explain the phylogenetic proximity between the two species (Pearson, 1937; Carvalho & Albert, 2011; Ota *et al*., 2014; Dagosta & de Pinna, 2019).

The genetic distance between *Odontostilbe pacaasnovos* and *O. pequira* ranged from 6.08% to 6.92%; while intraspecific genetic distance in *O. pequira* ranged from 0.00% to 4.05% and in *O. pacaasnovos* samples is 0.00% (Tab. 2). *Odontostilbe pequira* formed two well-supported clades, the Paraná-Paraguay clade and the Uruguay clade in the COI gene tree, separated by 19 mutational steps in the haplotype network. Recent studies of barcode DNA with fish have presented intraspecific variations that range from 0.00 to 10.91% (mean 0.35%; Ward *et al*., 2009), or 0.00 to 7.59% (mean 0.53%; Diaz *et al*., 2016). Also, species that have high intraspecific genetic distance (> 2.0%) have been recorded such as *Hoplias malabaricus* (Bloch) and *Potamotrygon motoro* (Müller & Henle) (Diaz *et al*., 2016), both species with a wide geographical distribution considered

by some authors as a complex of cryptic species that require a deeper revision (Marques *et al*., 2013; Pereira *et al.,* 2013; Diaz *et al*., 2016). *Odontostilbe pequira* requires further analysis to investigate if these genetic differences reveal different species or structured genetic populations (Sukumaran & Knowles, 2017; Leaché *et al*., 2019; Aguilar *et al*. 2019).

Differences in the number of lamellae of the olfactory rosette within Cheirodontinae was previously utilized by Chuctaya *et al*. (2018) to diagnose *O. avanhandava*, *O. microcephala* and *O. weitzmani*. Recently Abrahão *et al*. (2019) registered the presence of sexual dimorphism in several species of Characidae such as *Odontostilbe pequira*, *Axelrodia lindeae* Géry, *Microschemobrycon casiquiare* Böhlke, *Rhinobrycon negrensis* Myers, *Hemigrammus ulreyi* (Boulenger), *Paracheirodon axelrodi* (Schultz), *Heterocheirodon yatai*, (Casciotta, Miquelarena & Protogino), *Tyttobrycon dorsimaculatus* Géry, *T. hamatus* Géry, *T. marajoara* Marinho, Bastos & Menezes, and *T. shibattai* Abrahão, Pastana & Marinho. This difference in the number of lamellae would favor the increase in the area of the olfactory organ in males, allowing the detection of female pheromones (Bertelsen, 1951) or male-male detection related to cohort competition (Abrahão *et al*., 2019).

Recently, Mirande (2018) hypothesized the formation of the *Holoshesthes* clade, made up of *Odontostilbe pequira* and *Odontostilbe microcephala*. This result was based in a very poor taxonomic sample and should be considered with caution due to the low number of species evaluated within of Cheirodontinae and the low representation of molecular information (for poor taxonomic sampling and nomenclatural stability see Reis *et al*., 2019). In his analysis, molecular information of *O. microcephala* was missing, as well as it lacks morphological analysis of some species of *Odontostilbe* included in that study, such as *O. ecuadorensis* and *O. splendida*. The new species, however, does not

present the characteristics proposed by Eigenmann (1915) to diagnose the genus *Holoshesthes*, found in *Odontostilbe pequira* and some other *Odontostilbe* (Malabarba, 1998), that is the dentary teeth with at least five cusps, with the three central cusps larger, compressed and in a row forming a sharp cutting edge. The genus *Odontostilbe* has been proposed to be diagnosed by two putative synapomorphies, the second unbranched dorsal-fin ray is elongate and the unbranched pelvic-fin ray is elongate in males (Malabarba, 1998). Both characters are observable in *O. pequira*, but adult males of *O. pacaasnovos* did not present the extension of the unbranched rays of dorsal and pelvic-fins. This demonstrates that evolutionary changes in characters of tooth morphology and of sexual dimorphism associated to the elongation of fin rays may have changed in closed related species such as *O. pequira* and *O. pacaasnovos*, and need to be re-evaluated in more comprehensive phylogenies involving other characters and additional taxa. The new species is described herein in *Odontostilbe* since it belongs to a clade containing the type species of the genus, *O. fugitiva*, and other species currently assigned to the genus. We do not advance in this manuscript in the discussion of the validity of *Holoshesthes* as separate from *Odontostilbe* due to our limited taxon and gene sampling, and limit our comparisons to species putatively proximally related to the new species. The Parque Nacional do Pacaás Novos is one of areas more preserved in the Rondônia State and the main rivers of that Brazilian state have their headwaters located within of the park on the Serra dos Pacaás Novos (*e.g.* rios Jaciparaná, Candeias, Cautário, Pacaás Novos, Jamari, Outro Preto). In the park there are not roads, and the accesses to the headwaters only is possible by walking trails. Recent fieldwork in the upper rio Jamari, Jaciparaná, Candeias and Pacaás Novos yielded new species of *Ancistrus*, *Hypostomus*, and *Moenkhausia* that are awaiting a formal description. The first fish described in this region was *Characidium summus*, a putative endemic species from upper rio Pacaás Novos (Zanata & Ohara,

2015). The second species described, *Odontostilbe pacaasnovos* also seems to have a very restricted distribution. Several inventories in the lower portions of tributaries from rio Madeira in Rondônia (*i.e*. Jaciparaná, Jamari, Cautário, Pacaás Novos, Machado) failed to reveal the occurrence of *Odontostilbe pacaasnovos* in further localities beyond the type locality (*cf.* Santos, 1996; Queiroz *et al*., 2013a, Queiroz *et al*., 2013b; Casatti *et al*., 2013; Vieira *et al.,* 2016; Costa *et al.,* 2017). In lower portion of these rivers also occurs *O. fugitiva* and *O. nareuda* (Lima *et al*., 2013). The discovery of new species reinforces the importance of conservation and the need for a broad sampling in the headwaters that drain from the park.

5 COMPARATIVE MATERIAL

5.1 *Odontostilbe microcephala*

MCP 38311, 4 ex, 38.86–43.59 mm SL, Endorheic of Uruena River, drenaje of Bajo Paraná, Rosario de la Frontera, Salta, Bajo Paraná, Argentina, 25.00000 S 64.50000 W, 2 Mar 2001, Gladys Monasterio de Gonzo and Mario Mosqueira. USNM 321173, 5, (2 c&s) of 49 ex, 29.66–44.02 mm SL, Camatindi River, 8 km N Border Dept. Tarija, 40 km N Villamontes, Dept Chuquisaca. Rio Paraguay, Bolivia, 20.99271 S 63.39922 W, 2 Oct 1988, W. Starnes, L. Starnes, J. Sarmiento & R. Vasquez.

5.2 *Odontostilbe avanhandava*

LIRP 3239, 48.7 mm SL, ribeirão da Batalha, Fazenda Batalha (Pedro Queresma), rio Paranaíba, Paracatu, Minas Gerais State, Brazil, 17.423611° S, 47.453056° W, 27 Apr 2002, C. A. A. Figueiredo & E. S. S. Rego. FMNH 57871 (1, 62.1 mm SL), rio Tietê at Salto Avanhandava below the falls, Penápolis, São Paulo, 21.167648° S, 50.117562° W, 14 Sep 1908, J. Haseman.

5.3 *Odontostilbe weitzmani*

MZUSP 16851, 38.9 mm SL, male, rio Mogi Guaçu, Emas, Pirassununga, São Paulo, Brazil, 21.916666° S 47.383335° W, 22 Oct 1963, H. A. Britski. MZUSP 87947, (11, 42.5–50.5 mm SL), Itirapina, córrego da Lapa near the mouth and along the seawall of rock and the bridge on the road, 22.249918° S, 47.863376° W, 31 Jan 2002, E. N. Fragoso.

5.4 *Odontostilbe fugitiva*

ANSP 178908, 2 c&s (1 female, 1 male) of 12, Maynas, Loreto, Peru [lower rio Itaya] at bridge on Iquitos-Nauta highway, approximately 25 miles SSW of Iquitos. INPA 18465, 4 c&s (2 males, 2 females) of 73, Brazil, Amazonas, Ilha da Marchantaria. INPA 18506, 3 c&s (1 male, 1 female, 1 unsexed) of 50. Brazil, Amazonas, Paraná do Xiborena. INPA 18512, 1 male c&s, Brazil, Amazonas, lago Pirapora, Catalão. MZUSP 77844, 2 c&s, 36.9-40.0 mm SL, rio Pastaza, Pastaza, Ecuador. UFRGS 28507, 3, 27.89-33.54 mm SL, Ucayali basin, near of Contamana, Pucallpa, Perú, H. Ortega. UFRGS 28508, 8, 25.01-31.75 mm SL, Ucayali river, near of Contamana, Pucallpa, Ucayali basin, Perú, Set 2018, J. Chuctaya.

5.5 *Odontostilbe pulchra*

INHS 40101, 2 c&s (1 male, 1 female) of 20. Trinidad, Cumuto River, 5 km S Brazil on the road to Talparo. INHS 40081, 4 (2 females 32.6-33.3 mm SL, 2 unsexed 26.3-29.9 mm SL), Quare River, 1km E Valencia on road to Arima.

5.6 *Odontostilbe nareuda*

MZUSP 87759, (1 female 35.3 mm SL), Calama, Brazil, M. Goulding, 2 Feb 1981. FMNH 106433, 1 male c&s of 30, Bolivia, Pando, rio Madeira basin, creek at right

margin of rio Nareuda, ca. 3-4 km upstream of the mouth of rio Tahuamanu. UFRGS 21785, 1, 19.91 mm SL, rio Bavana Branco no Furo escadinha, Jurua, Carauari, Amazonas, 5° 12' 15.00" S 67° 15' 56.00" W, 17 Jun 2008, Malabarba, L.R.; Bertaco, V.A.; Jerep, F. C. & Carvalho, T. P. UFRGS 21687, 2, 18.63-18.74 mm SL, lago marginal ao rio Juruá no Porto de Carauari, Jurua, 4° 52' 52.00" S 66° 53' 42.00" W, 12 Jun 2008 Malabarba, L.R.; Bertaco, V.A.; Jereb, F. C.; Carvalho, T. P. & Motta, C. B. UFRGS 21731, 5, Varador da Fortuna, Comunidade da Fortuna (desemboca na igarapé Bavana Branco), afl. Jurua, 5° 9' 39.00" S 67° 15' 40.00" W, 16 Jun 2008, Malabarba, L.R.; Bertaco, V.A. & Araújo, R.

5.7 *Odontostilbe dierythrura*

MCP 38624, 2 c&s (1 male, 1 female) of 7, Bolívia, Cochabamba, rio Madeira, rio Ichilo basin, rio Samusabety, system Isiboro-Mamoré-Madeira.

5.8 *Odontostilbe euspilurus*

ANSP 143702, 2 c&s (1 male, 1 female) of 8, Peru, Cuzco/Madre de Dios, mouth of rio Carbon, below Atalaya on N/S road, above and below ford. MCP 38420, 13 ex, tributary of Rio Payamino, few km upstream from San Jose de Payamino, Napo, Equador.

5.9 *Odontostilbe pequira*

UFRGS 8641, 14 ex, Rio Uruguai, Uruguaiana, Rio Grande do Sul, Brazil. UFRGS 5589, 5 ex, stream of the Salso, Uruguaiana, Rio Grande do Sul, Brazil. UFRGS 13365, 22 ex, tributary of the left bank rio Soberbo, highway to the lago do Manso, Cuiabá, Mato Grosso, Brazil. UFRGS 13006, 99 ex, ribeirão Esmerial, Br-163. Mato Grosso, Brazil. MZUSP 21067, 1 male, 1 unsexed c&s of 53, Brazil, Paraná, rio Paraná below Sete Quedas, now Itaipu Reservoir area, CETESB. UFRGS 13507, 2 , rio Mutuca, próximo a ponte da MT-251.Mato Grosso, Cuiabá, Brazil, 15° 21' 55.7" S55° 57' 20.3" W, 25 Oct

2010, Baicere-silva, C.M.; Jerep, F.C.; Bertaco, V.A. & Carvalho, F.R. UFRGS 19414, 3, arroio Chancay, Lower Uruguay river, Província de Missiones, Argentina, 27° 29' 33.2" S 54° 40' 38.2", 21 Jun 2014 W Hirschmann, A.; Kasper, C.B.; Fenocchio, A. UFRGS 16647, 5, rio Santa Maria, praia de Areias Brancas dentro do município de Rosário do Sul, bacia do rio Uruguai Rosário do Sul, Rio Grande do Sul, Brazil, 30° 15' 02.6" S 54° 54' 41", Wingert, J.; Ferrer, J.; Miranda, J. P.; Giora, J. & Malabarba, L. R., UFRGS 28506, 3, Rio Garimpo na MS-080, Rio Negro, Mato Grosso do Sul, Brasil, 19°26'57.8"S 54°59'12.8"W, 13 Fev 2018, Carvalho, F. R.; Donin, L. M.

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FIGURES

FIGURE 1. *Odontostilbe pacaasnovos* sp. nov., (a) holotype, male, 27.8 mm SL, UFRGS 28509. (b) paratype, female, 29.1 mm SL, UFRGS 26296. Upper rio Jaciparaná, Parque Nacional Pacaás Novos, rio Madeira drainage**,** Rondônia**,** Brazil.

FIGURE 2. Dentition of *Odontostilbe pacaasnovos* sp. nov., paratype, UFRGS 26296: (upper) left side premaxilla and maxilla, (lower) left side dentary, lateral view. aa: anguloarticular; dt: dentary; mx: maxilla; ra: retroarticular; pm: premaxilla. Scale (bar: 0.5 mm**)**

FIGURE 3. Olfactory rosette slightly oval showing the lamellae of *Odontostilbe pacaasnovos* sp. nov., UFRGS 26296, paratypes: (a) female (27.3 mm), 14 lamellae (b) male (27.8 mm),18 lamellae.

FIGURE 4. *Odontostilbe pacaasnovos* sp. nov., upper rio Jaciparaná, Parque Nacional Pacaás Novos**,** rio Madeira drainage, Rondônia, Brazil.

FIGURE 5. Phylogenetic relationships of *Odontostilbe*: *O. fugitiva*, *O. microcephala*, *O. nareuda*, *O. paraguayensis*, *O. pequira*, and *Odontostilbe* ***pacaasnovos*** **sp. nov.,** using the Neighbor-Joining method, with the sum of branch length = 0.271. The analysis involved 17 nucleotide sequences with 539 positions in the final dataset of one mitochondrial loci (COI). Node numbers correspond to bootstrap value (1000 replicates). Jurua basin (JU), Madeira basin (MD), Paraguay basin (PA), Paraná basin (PR), Ucayali basin (UC), and Uruguay basin (UR),

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FIGURE 6. Haplotype network illustrating the genetic connectivity of COI haplotypes of *Odontostilbe pacaasnovos* sp. nov. (H1), *O. pequira* (H2, H3, H4, H5, H6, and H7), *O. microcephala* (H8), *O. paraguayensis* (H9), *Odontostilbe fugitiva* (H10), and *O. nareuda* (H11, H12). Each circle represents a unique haplotype with its size proportional to haplotype frequency, number of mutations is represented by number in the branches that connect the haplotypes.

FIGURE 7. Map showing the distribution of *Odontostilbe pacaasnovos* sp. nov in **the** rio Jaciparaná, **rio Madeira drainage,** Rondônia**, northern Brazil**. Type locality (red star).

FIGURE 8. Upper rio Jaciparaná, rio Madeira basin, Parque Nacional do Pacaás Novos, Campos Novos de Rondônia State, Rondônia, Brazil; type-locality of *Odontostilbe pacaasnovos* sp.n.

TABLE

TABLE 1. Morphometric data for *Odontostilbe pacaasnovos.* Number of individuals (N), mean, minimum (min), maximum (max) and standard deviation (SD) include values of the holotype (male).

TABLE 2. Genetic distance between sequences of COI of specimens of and *Odontostilbe pacaasnovos* sp. nov. (O.pac), *Odontostilbe pequira* (O.peq), *O. paraguayensis* (Opar), *O. fugitiva* (O.fug), *O. nareuda* (O.nar), and *O. microcephala* (O.mic). Jurua basin (JU), Madeira basin (MD), Paraguay basin (PA), Paraná basin (PR), Ucayali basin (UC), and Uruguay basin (UR),

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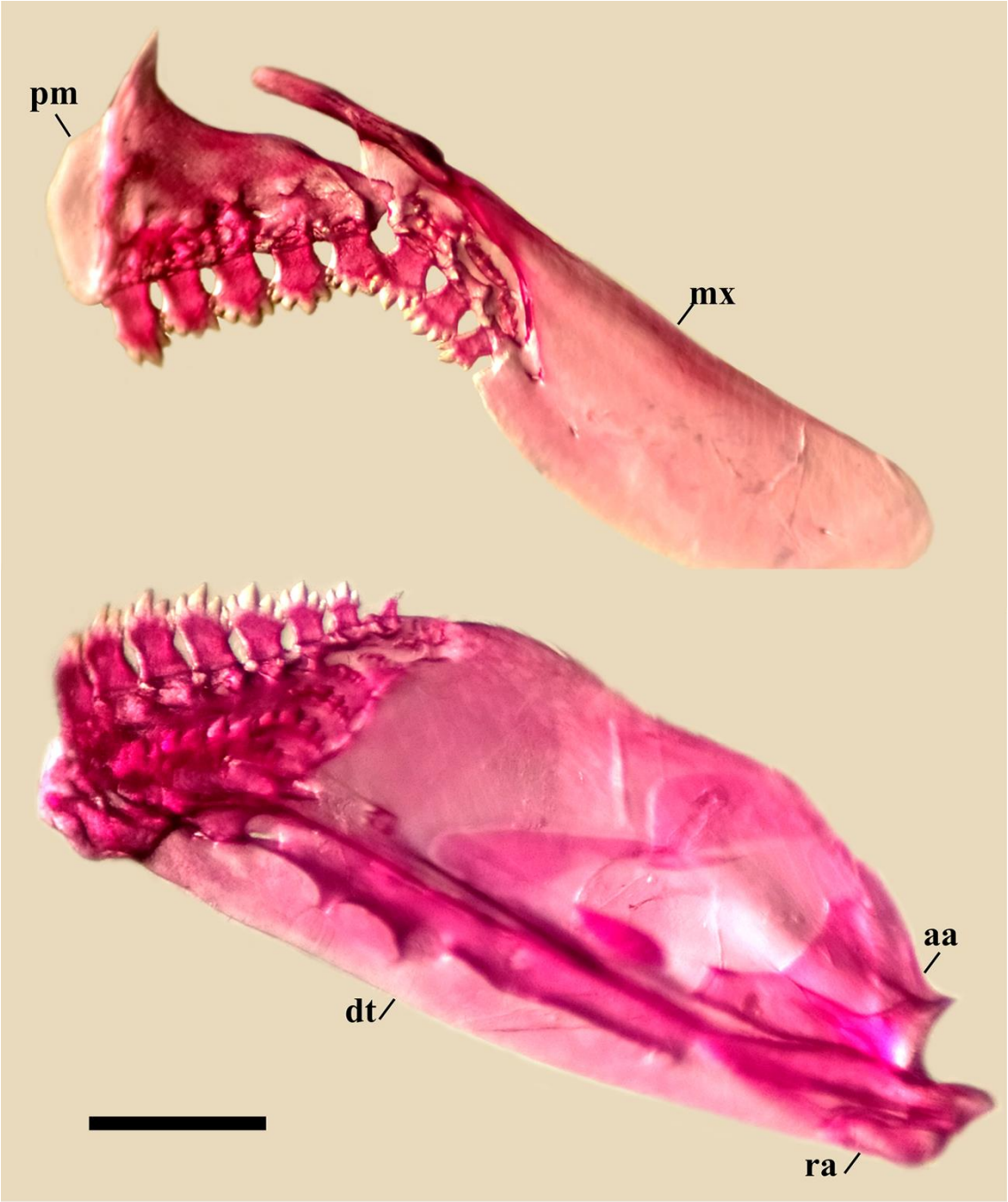

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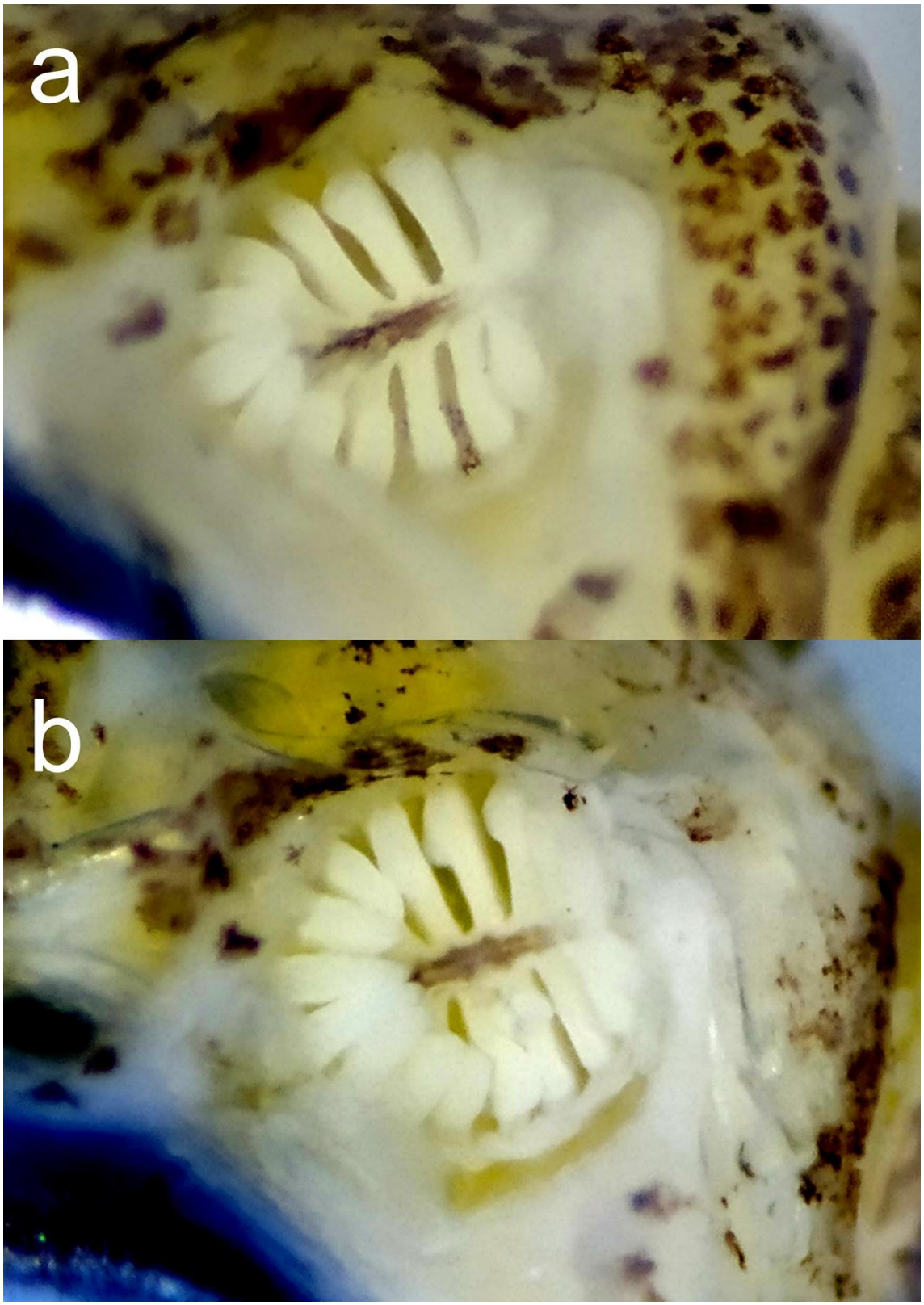

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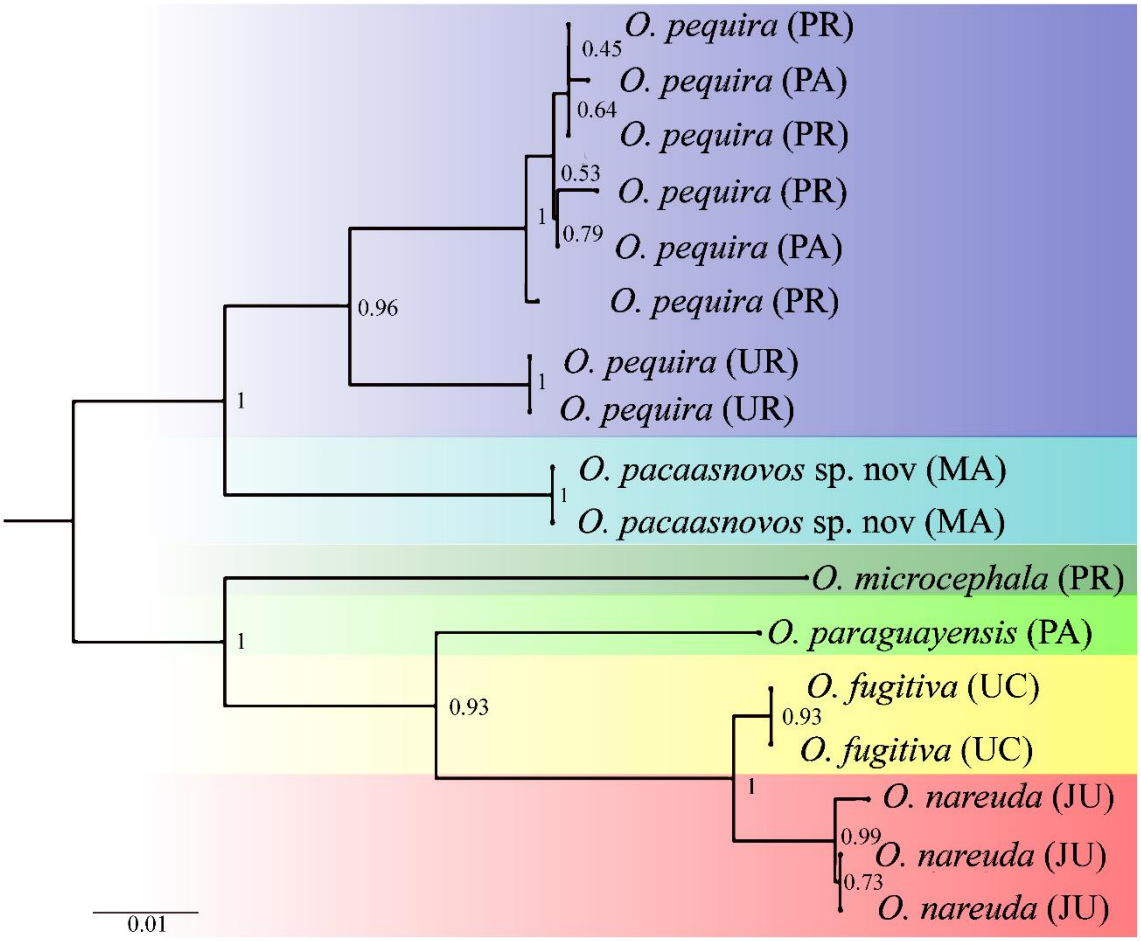

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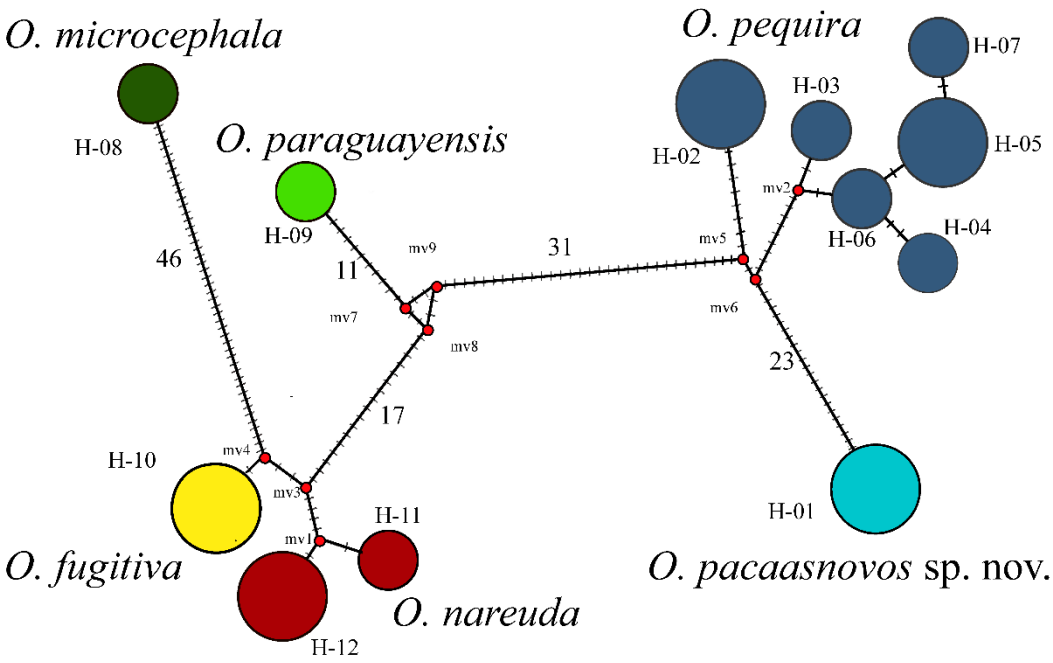

FIGURE 6. Haplotype network illustrating the genetic connectivity of COI haplotypes of *Odontostilbe pacaasnovos* sp. nov. (H1), *O. pequira* (H2, H3, H4, H5, H6, and H7), *O. microcephala* (H8), *O. paraguayensis* (H9), *Odontostilbe fugitiva* (H10), and *O. nareuda* (H11, H12). Each circle represents a unique haplotype with its size proportional to haplotype frequency, number of mutations is represented by number in the branches that connect the haplotypes.

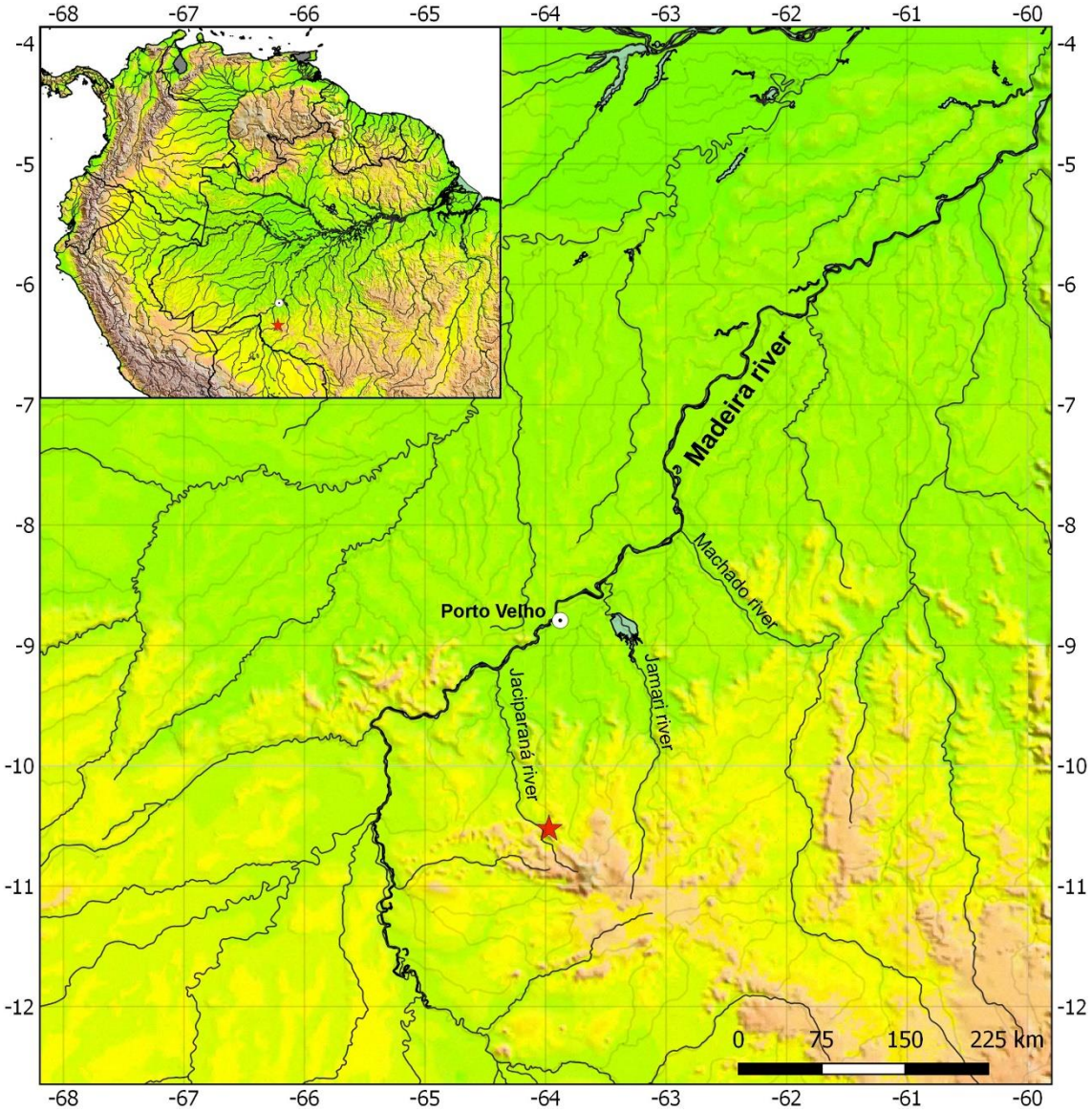

FIGURE 7. Map showing the distribution of *Odontostilbe pacaasnovos* sp. nov in the rio Jaciparaná, rio Madeira drainage**,** Rondônia**,** northern Brazil**.** Type locality (red star).

FIGURE 8. Upper rio Jaciparaná, rio Madeira basin, Parque Nacional do Pacaás Novos, Campos Novos de Rondônia State, Rondônia, Brazil; type-locality of *Odontostilbe pacaasnovos* sp.n.

TABLE 1. Morphometric data for *Odontostilbe pacaasnovos.* Number of individuals (N), mean, minimum (min), maximum (max) and standard deviation (SD) include values of the holotype (male).

		Male				Female				
	Holotype	**N**	**Min**	**Max**	**Mean**	**SD**	**N**	**Min**	**Max**	**Mean**	**SD**
Standard length (mm)	27.8	15	26.26	31.51	27.7	-	19	25.14	31.3	27.5	-
Percents of Standard Length											
Snout to anal-fin origin	62.6	15	60.7	63.0	62.0	0.7	19	61.1	65.2	62.8	1.2
Snout to dorsal-fin origin	49.9	15	49.9	52.2	51.0	0.7	19	49.6	53.5	51.5	1.2
Snout to pelvic-fin origin	47.1	15	44.0	48.7	46.3	1.2	19	45.1	48.7	46.9	1.1
Snout to pectoral-fin origin	24.6	15	22.7	25.0	24.1	0.7	19	22.2	27.2	24.2	1.1
Dorsal-fin origin to caudal fin distance	55.1	15	51.2	55.5	53.5	1.1	19	50.7	54.0	52.6	0.9
Orbit posterior margin to dorsal-fin origin	36.8	15	36.7	39.7	38.0	1.1	19	37.3	40.6	38.7	0.9
Anal-fin base length	26.7	15	26.0	28.5	27.2	0.8	19	25.7	28.6	27.1	0.8
Length of caudal peduncle	15.4	15	14.5	17.7	15.9	1.0	19	14.8	17.8	16.1	1.0
Depth of caudal peduncle	10.3	15	8.7	11.6	10.3	0.7	19	7.8	11.9	10.6	1.0
Body depth at dorsal fin	28.5	15	26.4	29.7	28.4	0.8	19	26.7	31.7	29.4	1.4
Dorsal-fin length	25.4	15	23.7	27.5	25.9	1.2	19	24.8	28.2	26.5	1.0
Pelvic-fin length	17.4	15	15.5	18.5	17.0	0.8	19	13.9	17.7	16.5	0.9
Pectoral-fin length	21.5	15	17.9	22.9	20.7	1.6	19	18.7	22.7	20.4	1.2
Head length	25.0	15	23.3	26.3	24.6	0.8	19	23.1	26.8	24.5	1.1
Percents of Head Length											
Snout length	6.2	15	5.0	6.7	6.0	0.5	19	4.9	6.9	5.9	0.6
Upper jaw length	9.1	15	8.1	10.0	9.0	0.5	19	8.1	10.1	9.1	0.5
Horizontal orbit diameter	9.3	15	8.7	10.8	9.6	0.6	19	9.2	11.8	10.0	0.6
Interorbital width	6.8	15	6.5	7.5	7.0	0.3	19	6.4	8.3	7.2	0.4

TABLE 2. Genetic distance between sequences of COI of specimens of and *Odontostilbe pacaasnovos* sp. nov. (O.pac), *Odontostilbe pequira* (O.peq), *O. paraguayensis* (Opar), *O. fugitiva* (O.fug), *O. nareuda* (O.nar), and *O. microcephala* (O.mic). Jurua basin (JU), Madeira basin (MD), Paraguay basin (PA), Paraná basin (PR), Ucayali basin (UC), and Uruguay basin (UR),

	Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*O.pac*_UFRGS26296.1_MD																	
2	*O.pac*_UFRGS26296.2_MD	0.00																
3	*O.peq*_UFRGS19414_UR	6.30	6.30															
4	*O.peq*_UFRGS16647_UR	6.30	6.30	0.00														
5	*O.peq*_KU288885.1_PR	6.08	6.08	3.65	3.65													
6	*O.peq*_KU288855.1_PR	6.92	6.92	4.05	4.05	0.75												
7	*O.peq*_KU288856.1_PR	6.29	6.29	3.85	3.85	0.56	0.56											
8	*O.peq*_KU288858.1_PR	6.29	6.29	3.85	3.85	0.56	0.56	0.00										
9	*O.peq*_UFRGS28506_PA	6.49	6.49	3.65	3.65	0.37	0.37	0.19	0.19									
10	*O.peq*_UFRGS13507_PA	6.49	6.49	4.05	4.05	0.75	0.75	0.19	0.19	0.37								
11	*O.mic*_CI-FML 7335_PR	11.98	11.98	11.05	11.05	11.49	12.42	11.72	11.72	11.95	11.72							
12	*O.par*_UFRGS13584_PA	10.77	10.77	10.54	10.54	10.75	11.66	11.43	11.43	11.21	11.66	11.88						
13	*O.fug*_UFRGS28508_UC	11.00	11.00	11.22	11.22	10.98	11.89	11.66	11.66	11.43	11.89	9.65	6.51					
14	*O.fug*_UFRGS28507_UC	11.00	11.00	11.22	11.22	10.98	11.89	11.66	11.66	11.43	11.89	9.65	6.51	0.00				
15	*O.nar*_UFRGS21731_JU	11.91	11.91	12.61	12.61	11.89	12.83	12.59	12.59	12.36	12.83	10.54	6.93	1.51	1.51			
16	*O.nar*_UFRGS21785_JU	11.91	11.91	12.15	12.15	11.43	12.36	12.13	12.13	11.89	12.36	10.54	6.93	1.51	1.51	0.37		
17	*O.nar*_UFRGS21687_JU	11.91	11.91	12.15	12.15	11.43	12.36	12.13	12.13	11.89	12.36	10.54	6.93	1.51	1.51	0.37	0.00	

Considerações Finais

Erros de identificação são comuns envolvendo as espécies de Cheirodontinae, relacionados ao fato de que grande parte das espécies de Cheirodontinae só pode ser reconhecida pela observação das características sexuais em machos maduros. Desde Malabarba (1998) com estudos de relações filogenéticas com dados morfológicos até o recente trabalho de Melo et al. 2022, com dados genômicos com UCEs, as relações entre os gêneros de Cheirodontinae apresentaram mudanças em sua localização na filogenia, em muitos casos, resultando em gêneros polifiléticos como *Odontostilbe* e *Serrapinnus*. Abordamos este grande problema sobre as relações filogenéticas de Cheirodontinae. Inicialmente, realizamos análises de delimitação de espécies com o objetivo de encontrar linhagens independentes dentro de espécies válidas. Nossas análises corroboraram esta hipótese, com 9 novas espécies potenciais. Um próximo passo foi abordar este grande dilema, em hipóteses de relações filogenéticas com diferentes tipos de dados (moleculares vs morfológicos). Para isso, integramos esses dois tipos de evidências e testamos com diferentes metodologias (análise de parcimônia e modelos). Nossas topologias usando as diferentes evidências mostraram que os gêneros dentro da subfamília estão agrupados em 8 clados (Protocheirodontini, Pseudocheirodontini, Cheirodontini, Amblystilbini, Holoshesthini, Compsurini, Prodontocharacini, Macropsobryconini), esses clados formam grupos monofiléticos fortemente suportados. Atualmente pouco se sabe sobre o processo de diversificação de Cheirodontinae na região Neotropical, sendo uma subfamília amplamente distribuída, é uma subfamília modelo para estudos biogeográficos. Usando nossos dados integrados (morfológicos e moleculares), estimamos a idade desta subfamília e propusemos a reconstrução da área ancestral e hipóteses de distribuição biogeográfica em toda a sua distribuição. Descobrimos que a espécie se diversificou de seu grupo irmão Aphyocharacine há 28 Ma, e sua área de

origem foi a Proto-Amazônia, e por processos de dispersão, atingiu regiões cis-andinas na América Central e rios costeiros da encosta do Pacífico em Chile. Adicionalmente, estudamos a influência do tamanho e capacidade de inseminação de Cheirodontinae em seu processo de diversificação. Constatamos que esses caracteres não influenciaram sua diversificação e que o caráter inseminador apareceu de forma independente 7 vezes na história dos Cheirodontinae. Ressaltamos que há muitos estudos a serem realizados dentro da subfamília, várias espécies e gêneros a serem descritos, além de conhecer algumas relações dentro da subfamília que ficaram sem solução devido à falta de dados. Os taxonomistas estão em constante luta contra o tempo, muitas espécies são extintas a cada dia, sem conhecer sua identidade taxonômica, devido ao crescente impacto que os ambientes aquáticos continentais estão sofrendo.

Referencias

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