



Rede de Biodiversidade e Biotecnologia da Amazônia Legal

**TAXONOMIA INTEGRATIVA DAS ESPÉCIES E POPULAÇÕES DO
CLADO *HYPHESSOBRYCON* “ROSY TETRA” QUE OCORREM NA
REGIÃO HIDROGRÁFICA ATLÂNTICO NORDESTE OCIDENTAL E
DRENAGENS ADJACENTES**

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São Luís – MA

2020

ERICK CRISTOFORE GUIMARÃES

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Tese apresentada ao Programa de Pós-graduação em Biodiversidade e Biotecnologia da Amazônia Legal - BIONORTE, como requisito parcial para a obtenção do título de Doutor em Biodiversidade e Biotecnologia

Orientador: Prof. Dr.: Felipe Polivanov Ottoni

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LISTA DE SIGLAS E ABREVIATURAS

CICCAA - Coleção Ictiológica do Centro de Ciências Agrárias Ambientais, da Universidade Federal do Maranhão.

CM - Carnegie Museum of Natural History, Pittsburgh, Pennsylvania, U.S.A; and

CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico – Ministério da Ciência e Tecnologia.

CPUFMA - Coleção de Peixes da Universidade Federal do Maranhão

FAPEMA - Fundação de Amparo à Pesquisa e Desenvolvimento Científico do Maranhão.

IBAMA – Instituto Brasileiro de Meio Ambiente

MCP - Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre

MHNM - Museo Nacional de História Natural y Antropología, Zoología, Sección Ictiología, Ministerio de Educación y Cultura, Montevideo, Uruguay

SISBIO – Sistema de informação da Biodiversidade

UFRJ - Coleção Ictiológica do Instituto de Biologia, Universidade Federal do Rio de Janeiro. Rio de Janeiro

USMN - Smithsonian Institution National Museum of Natural History, Washington D.C., U.S.A

APRESENTAÇÃO

O conteúdo da tese será apresentado em capítulos, listados abaixo:

A tese está organizada em três capítulos: o primeiro - **“*Hyphessobrycon* “rosy tetra”** - Traz um breve comentário e histórico sobre o atual conhecimento taxonômico e sistemático do grupo; o segundo – **“A new cryptic species of *Hyphessobrycon* Durbin, 1908 (Characiformes, Characidae) from the Eastern Amazon, revealed by integrative taxonomy”** descreve uma nova espécie do gênero *Hyphessobrycon* para a bacia do Mearim no Estado do Maranhão (Publicado em junho de 2019 na *Zoosystematics and Evolution* 95 (2) 2019, 345–360 (Anexo 1); e o terceiro – **“Integrative taxonomy reveals two new cryptic species of *Hyphessobrycon* Durbin, 1908 (Teleostei: Characidae) from the Maracaçumé and middle Tocantins River basins, Eastern Amazon region”** descreve duas novas espécie do gênero *Hyphessobrycon* para as bacias do Maracaçumé e Tocantins nos Estados do Maranhão e Pará respectivamente (Aceite definitivo em julho de 2020 na *European Journal Taxonomy* (Anexo 2).

RESUMO

Taxonomia integrativa das espécies e populações do clado *Hyphessobrycon* “rosy tetra” que ocorrem na Região Hidrográfica Atlântico Nordeste Ocidental e drenagens adjacentes

Erick Cristofore Guimarães

Orientador: Felipe Polivanov Ottoni

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Resumo da tese submetida ao Programa de Pós-graduação em Biodiversidade e Biotecnologia da Amazônia Legal – PPG – BIONORTE, como requisito parcial para a obtenção do título de Doutor em Biodiversidade e Biotecnologia.

A Ordem Characiformes é um dos componentes da fauna aquática com o maior número de espécies, com aproximadamente 3.700 espécies válidas formada por quatro famílias africanas e 20 neotropicais. Dentre os Characiformes, a Família Characidae é a mais diversa e problemática em termos taxonômicos, com cerca de 1.200 espécies (representando aproximadamente 58% das espécies entre os Characiformes), e a quarta família mais diversa de peixes, após Cyprinidae, Cichlidae e Gobiidae. Dentro da família Characidae, *Hyphessobrycon* é um bom exemplo de grupo problemático, onde as relações dentro das espécies desse gênero se apresentam ainda um tanto inconclusivas. *Hyphessobrycon* é um dos gêneros mais ricos em espécies de Characidae, atualmente compreendendo aproximadamente 160 espécies válidas. São amplamente distribuídos ao longo das bacias hidrográficas da região Neotropical, do sul do México até bacia do rio da Prata no nordeste da Argentina. Uma maneira de superar a taxonomia confusa de grupos problemáticos, ter precisão na identificação de espécies e estimativas de diversidade de grupos de espécies é usar critérios operacionais para delimitação de espécies. O aprimoramento do conhecimento sistemático, taxonômico e de distribuição da Ictiofauna é de fundamental relevância no contexto atual do desenvolvimento brasileiro, principalmente para o campo da conservação das espécies. Os ambientes naturais sofrem com a acelerada destruição e consequente extinção e perda de espécies conhecidas e até mesmo de espécies ainda não conhecidas para a ciência. Sendo assim, o objetivo principal deste projeto é ampliar o

conhecimento taxonômico referente às espécies e populações do clado *Hyphessobrycon* “rosy tetra” que ocorrem na região hidrográfica Atlântico Nordeste Ocidental e drenagens adjacentes com base em diferentes e independentes métodos de delimitação de espécies, além de utilizar esses dados para tomar decisões taxonômicas sobre o grupo.

Palavras-chave: delimitação de espécies. DNA-barcoding. *Hyphessobrycon sensu stricto*

ABSTRACT

Integrative taxonomy of the species and populations of the *Hyphessobrycon* “rosy tetra” clade that occur in the Região Hidrográfica Atlântico Nordeste Ocidental and adjacent drainages

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Abstract of the thesis submitted to the Postgraduate Program in Biodiversity and Biotechnology of the Legal Amazon – PPG – BIONORTE, as a partial requirement to obtain a PhD in Biodiversity and Biotechnology

The Order Characiformes is one of the components of the aquatic fauna with the largest number of species, with approximately 3,700 valid species formed by four African and 20 Neotropical families. Among the Characiformes, the Characidae Family is the most diverse and problematic in taxonomic suits, with about 1,200 species (representing approximately 58% of the species among Characiformes), and the fourth most diverse family of fish, after Cyprinidae, Cichlidae and Gobiidae. Within the Characidae family, *Hyphessobrycon* is a good example of a problematic group, where relations within species of this genus are still somewhat inconclusive. *Hyphessobrycon* is one of the richest genera in species of Characidae, currently comprising approximately 160 valid species. They are widely distributed along the hydrographic basins of the Neotropical region, from southern Mexico to the Río de la Plata basin in northeastern Argentina. One way to overcome the confusing taxonomy of problem groups, to be precise in identifying species and to estimate diversity of species groups is to use operational criteria for species

delimitation. The improvement of the systematic, taxonomic and distribution knowledge of the ichthyofauna is of fundamental relevance in the current context of Brazilian development, mainly for the field of species conservation. Natural environments suffer from the accelerated destruction and consequent extinction and loss of known species and even species not yet known to science. Therefore, the main objective of this project is to expand the taxonomic knowledge regarding the species and populations of the Hyphessobrycon clade “rosy tetra” that occur in the Western Northeast Atlantic hydrographic region and adjacent drainages based on different and independent species delimitation methods, in addition to use this data to make taxonomic decisions about the group.

Key words: DNA-barcoding. *Hyphessobrycon sensu stricto*. species delimitation

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CAPÍTULO I

Hyphessobrycon “rosy tetra”: introdução geral

Este capítulo é introdutório à tese e não será submetido à publicação

1. Introdução

A América do Sul é o principal continente do mundo quando falamos sobre biodiversidade de peixes, sendo o continente com a ictiofauna mais diversa, com estimativas atuais de mais de 9.100 espécies válidas ocorrendo em ambientes de água doce e regiões marinhas próximas à costa (27% das espécies de peixes do mundo) (REIS et al., 2016). A ictiofauna de água doce do continente é mais rica do que a ictiofauna marinha, possuindo cerca de 5.160 espécies descritas (cerca de um terço de todos dos peixes de água doce do mundo). A publicação da Lista de dos Peixes de Água Doce que ocorrem na América do Sul e América Central (CLOFFSCA) listou cerca de 4.500 espécies válidas para a região Neotropical, estimando que ainda havia cerca de 1.550 espécies não descritas (REIS et al., 2003). Entretanto, novas estimativas apontam que a diversidade da ictiofauna dulcícola, considerando apenas a América do Sul, possa ser muito maior, na ordem de oito a nove mil espécies (REIS et al., 2016).

O Brasil concentra as maiores redes hidrográficas da região Neotropical, as quais apresentam elevada biodiversidade aquática, compreendendo 21% das espécies de peixes de água doce do mundo (REIS et al., 2003). Sendo assim, estima-se que ocorram aproximadamente 2.600 espécies dulcícolas de peixes em território brasileiro (REIS et al., 2003). A maior parte dessa diversidade corresponde a espécies de pequeno e médio porte, que estão distribuídas principalmente em riachos (REIS et al., 2016).

A Ordem Characiformes é um dos componentes da fauna aquática com o maior número de espécies, compreendendo aproximadamente 3.700 espécies válidas, ocorrendo nas regiões biogeográficas Etiópica e Neotropical (FRICKE et al., 2020). A região Neotropical inclui 20 famílias dessa ordem (FRICKE et al., 2020), distribuídas do sudoeste dos Estados Unidos ao norte da Patagônia, habitando rios e riachos, ambientes lóticos e lênticos, águas límpidas e turvas (FRICKE et al., 2020). Dentro de Characiformes, destaca-se Characidae, por ser a família mais diversa e a mais problemática em termos taxonômicos, com cerca de 1.200 espécies (representando cerca de 32% das espécies de Characiformes). Além disso, Characidae é a quarta família mais diversa de peixes, atrás apenas de Gobiidae, Cichlidae e Cyprinidae (FRICKE et al., 2020).

Hyphessobrycon Durbin, 1908, é um dos gêneros com maior número de espécies tanto da subfamília Stethaprioninae quanto da família Characidae, atualmente compreendendo aproximadamente 160 espécies válidas (FRICKE et al., 2020),

amplamente distribuídas ao longo das bacias hidrográficas da região Neotropical, com maior diversidade na bacia Amazônica (GUIMARÃES et al., 2018).

As relações entre as espécies de *Hyphessobrycon* é um bom exemplo de grupo confuso taxonomicamente de Characidae. Dados extensivos mostram que o gênero não é monofilético, assim como a relação interna de seus membros ainda não está resolvida (GUIMARÃES et al., 2018). *Hyphessobrycon* foi diagnosticado por Eigenmann (1917) com base em uma combinação artificial de estados de caráter, tais como: a presença de uma nadadeira adiposa; maxilar com poucos dentes ou nenhum; linha lateral incompleta; terceiro osso infraorbital não em contato com o canal sensorial do pré-opérculo; pré-maxilar com duas séries de dentes; e lobos da nadadeira caudal sem escamas na base. No entanto, descrições de novas espécies continuaram a seguir esta combinação artificial (ver: FRICKE et al., 2020). Além disso, alguns grupos de espécies dentro do gênero foram propostos de forma artificial, sem o acompanhamento de uma análise filogenética, com base em combinações de estados de caracteres, principalmente relacionados ao padrão de colorido (por exemplo: WEITZMAN; PALMER, 1997).

Um desses grupos de espécies foi denominado como “grupo F” por Géry (1977), sendo definido pela presença de uma mancha marrom escura ou preta na nadadeira dorsal e ausência de uma faixa ao longo do corpo. Weitzman e Palmer (1997) propuseram o nome clado *Hyphessobrycon* “rosy tetra” para este grupo proposto por Géry (1977), incluindo aproximadamente 30 espécies de *Hyphessobrycon* e algumas outras espécies pertencentes a outros gêneros provavelmente relacionados a essas espécies de *Hyphessobrycon*, segundo esses autores. Esses autores também sugeriram a presença da mancha negra na nadadeira dorsal como um dos principais caracteres deste agrupamento. Entretanto, eles também afirmaram que esta mancha estava ausente em algumas de suas espécies.

Carvalho e Malabarba (2015) propuseram o grupo denominado *Hyphessobrycon sensu stricto*, incluindo a espécie tipo do gênero [*Hyphessobrycon compressus* (Meek 1904)] e espécies do clado *Hyphessobrycon* “rosy tetra” *sensu* Weitzman e Palmer (1997), diagnosticado pela posição do aparelho weberiano horizontal ascendente através da margem dorsal do opérculo, presença de mancha marrom escura ou preta na nadadeira dorsal e a ausência de uma faixa preta ao longo do corpo. Entretanto, *Hyphessobrycon sensu stricto* possui uma composição mais restrita do que a do Clado *Hyphessobrycon* “rosy tetra” *sensu* Weitzman e Palmer (1997), compreendendo cerca de 25 espécies. Algumas dessas espécies foram recentemente descritas para o clado *Hyphessobrycon*

“rosy tetra”, tais como *Hyphessobrycon dorsalis* Zarske, 2014, *H. jackrobertsi* Zarske, 2014, *H. paepkei* Zarske, 2014 e *H. pando* Hein, 2009, mas de acordo com Carvalho e Malabarba (2015) seus status taxonômicos são incertos, e suas descrições muito pouco informativas.

Além disso espécies, *Hyphessobrycon piorskii* Guimarães, Brito, Feitosa, Carvalho-Costa & Ottoni 2018 e *Hyphessobrycon caru* Guimarães, Brito, Feitosa, Carvalho-Costa & Ottoni 2018 também foram recentemente descritas, sendo as primeiras espécies registradas do grupo para drenagens maranhenses. Assim como foram as primeiras espécies desse grupo descrita a partir da utilização de diferentes critérios operacionais para delimitação de espécies, convergindo para a proposta atual de uma taxonomia integrativa (ver GOLDESTEIN; DESALLE, 2010; PADIAL et al., 2010).

Três artigos publicados recentemente (por exemplo, CASTRO-PAZ et al. 2014; GUIMARÃES et al. 2018, 2019) aplicaram abordagens de delimitação de espécies moleculares para investigar a diversidade de espécies no clado “rosy tetra” de *Hyphessobrycon*, e todos concordaram que sua taxonomia deveria ser melhor investigado, uma vez que inclui espécies enigmáticas. Da mesma forma, outros estudos, incluindo outros grupos e gêneros de Characiformes, revelaram uma alta diversidade críptica (por exemplo, PEREIRA et al. 2011; MELO et al. 2014; BENINE et al. 2015; BENZAQUEM et al. 2015; MELO et al. 2016a, b, c; GARCÍA-MELO et al. 2019).

Além da ampla distribuição e da presença de espécies enigmáticas, a história taxonômica confusa desse clado é um dos principais desafios ao estudar sua diversidade e relacionamentos. As espécies-tipo de *Hemigrammus* Gill 1858, *H. unilineatus* (Gill 1858) e *Pristella* Eigenmann 1908, *P. maxillaris* (Ulrey 1894), têm sido historicamente consideradas estreitamente relacionadas ou pertencentes ao clado “rosado-tetra”, que também inclui as espécies do tipo *Hyphessobrycon*, *H. compressus* (Meek 1904) (Weitzman & Palmer 1997; Carvalho & Malabarba 2015). Assim, *Hyphessobrycon* pode ser um sinônimo putativo de *Hemigrammus* ou um novo gênero (MIRANDE, 2010). No entanto, mais estudos sobre a sistemática das espécies atualmente atribuídas a *Hyphessobrycon* e gêneros relacionados próximos ainda são necessários para esclarecer esta questão (GUIMARÃES et al. 2018, 2019).

2. Objetivos

2.1. Objetivo geral

Realizar um estudo taxonômico das espécies do clado *Hyphessobrycon* “rosy tetra” que ocorrem na região hidrográfica Atlântico Nordeste Ocidental e drenagens adjacentes, com base em diferentes e independentes métodos de delimitação de espécies, e utilizar esses dados para se tomar decisões taxonômicas relacionadas as espécies do grupo.

2.2. Objetivos específicos

- 1- Realizar um estudo com base em taxonomia integrativa das espécies do clado *Hyphessobrycon* “rosy tetra” que ocorrem na região hidrográfica Atlântico Nordeste Ocidental e drenagens adjacentes;
- 2- Comparar a sensibilidade e eficácia de cada método de delimitação de espécies empregado para o grupo estudado;
- 3- Sequenciar e disponibilizar segmentos parciais do gene mitocondrial Citocromo Oxidase subunidade I (COI) dos exemplares analisados durante o projeto em bancos de dados on-line (por exemplo, Genbank e Bold);
- 4- Descrever possíveis novas espécies do grupo estudado e revelar espécies crípticas;
- 5- Publicar os resultados obtidos durante o projeto em periódicos científicos, preferencialmente, de livre acesso, e em encontros científicos.

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CAPÍTULO II

A new cryptic species of *Hyphessobrycon* Durbin, 1908 (Characiformes, Characidae) from the Eastern Amazon, revealed by integrative taxonomy

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A new cryptic species of *Hyphessobrycon* Durbin, 1908 (Characiformes, Characidae) from the Eastern Amazon, revealed by integrative taxonomy

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Abstract

Hyphessobrycon caru sp. nov. is described based on five different and independent methods of species delimitation, making the hypothesis of this new species supported by an integrative taxonomy perspective. This new species has a restricted distribution, occurring just in the upper Pindaré river drainage, Mearim river basin, Brazil. It is a member of the rosy tetra clade, which is characterized mainly by the presence of a dark brown or black blotch on dorsal fin and absence of a midlateral stripe on the body. *Hyphessobrycon caru* sp. nov. is distinguished from the members of this clade mainly by the shape of its humeral spot, possessing few irregular inconspicuous vertically arranged chromatophores in the humeral region, or sometimes a very thin and inconspicuous humeral spot, and

other characters related to teeth count, and color pattern. The phylogenetic position of the new species within the rosy tetra clade was based on molecular phylogenetic analysis using sequences of the mitochondrial gene cytochrome oxidase subunit 1. In addition, a new clade (here termed *Hyphessobrycon micropterus* clade) within the rosy tetra clade is proposed based on molecular data, comprising *H. caru* sp. nov., *H. micropterus*, *H. piorskii*, and *H. simulatus*, and with *H. caru* sp. nov. and *H. piorskii* recovered as sister species. Our results suggest cryptic speciation in the rosy tetra clade and, more specifically, in the *H. micropterus* clade. We recommend the use of integrative taxonomy for future taxonomic revisions and species descriptions when dealing with species complexes and groups containing possible cryptic species.

Key Words: bPTP, DNA barcoding, rosy tetra clade, species complex, Stethaprioninae

Introduction

Hyphessobrycon Durbin, 1908 is a species-rich characid genus comprising about 160 valid species (Fricke et al. 2019). It is widely distributed along the river basins of the Neotropical region, from southern Mexico to the La Plata River basin in northeastern Argentina (Carvalho and Malabarba 2015; García-Alzate et al. 2017; Guimarães et al. 2018). The genus was first proposed as a subgenus of *Hemigrammus* Gill, 1858 by Durbin in Eigenmann (1908), differing from the latter only by the absence of scales covering the caudal-fin. *Hyphessobrycon* was reviewed by Eigenmann (1918, 1921) in a work which still constitutes the most comprehensive revisionary studies on the genus. The large number of species included within *Hyphessobrycon* and the poor knowledge of the alpha and beta-taxonomy of species and species groups are among the major challenges for a more comprehensive taxonomic study and phylogenetic analyses of the genus. It is widely known that *Hyphessobrycon* does not constitute a monophyletic group (Weitzman and Palmer 1997a; Mirande 2010, 2018; Oliveira et al. 2011; Carvalho and Malabarba 2015; Carvalho et al. 2017; Moreira and Lima 2017; Betancur-R. et al. 2018; Guimarães et al. 2018). Nevertheless, groups of species have been proposed based primarily on similarities of color pattern and other external features (e.g. Weitzman and Palmer 1997a; García-Alzate et al. 2008; Moreira and Lima 2017). Some of them are probably merely artificial operational assemblages to aid species identification, whereas others represent potential monophyletic groups, delimited by exclusive character states (e.g. Castro-Paz et al. 2014; Carvalho and Malabarba 2015; Guimarães et al. 2018).

Several genetic studies focusing on characoid fishes, such as *Astyanax* Baird & Girard, 1854 (e.g. Ornelas-Garcia et al. 2008), *Caenotropus* Günther, 1864 (e.g. Melo et al. 2014), *Chilodus* Müller & Troschel, 1844 (e.g. Melo et al. 2014), *Curimatopsis* Steindachner, 1876 (e.g. Melo et al. 2016a), *Gymnocorymbus* Eigenmann, 1908 (e.g. Benine et al. 2015), *Hyphessobrycon* (e.g. Castro-Paz et al. 2014, Guimarães et al. 2018), *Piabina* Reinhardt, 1867 (e.g. Pereira et al. 2011), *Prochilodus* Agassiz, 1829 (e.g. Melo et al. 2016b), *Nannostomus* Günther, 1872 (e.g. Benzaquem et al. 2015) and *Tetragonopterus* Bleeker, 1863 (e.g. Melo et al. 2016c) have evidenced that some species may exhibit large discontinuities in their geographic distribution patterns, with high genetic divergences, but little morphological variability among geographically isolated lineages. These results suggest that these groups may represent species complexes or cryptic species, that is, they might even including morphologically quite similar or undistinguishable species that are hidden and erroneously classified (Brown et al. 1995; Bickford et al. 2006; Adams et al. 2014; Souza et al. 2018). Studies relying solely on morphology may be inadequate in recognizing species within groups including cryptic species (Guimarães et al. 2018). Integrative studies, using more than one criteria, such as character-based, tree-based, genetic distance and coalescent-based approaches, especially including molecular data, are useful and powerful for the recognition of hidden and/or possible new species in such species complexes (Sytsma and Schaal 1985; Bickford et al. 2006; Goldstein and Desalle 2010; Padial et al. 2010; Adams et al. 2014; Costa-Silva et al. 2015; Souza et al. 2018; Ottoni et al. 2019).

In this context of integrative taxonomy, the present study aims to investigate the diversity within the rosy tetra clade sensu Weitzman and Palmer (1997a). This clade comprises around 30 species, including some species of *Hyphessobrycon* and other allied species, that are appreciated as aquarium fishes due to their attractive color patterns (e.g. Weitzman and Palmer 1997a, 1997b, 1997c, 1997d; Zarske 2008; Hein 2009; Guimarães et al. 2018).

This group has had its composition and name changed over the last decades, and a detailed taxonomic history is presented by Weitzman and Palmer (1997a). Two previous papers (e.g. Castro-Paz et al. 2014; Guimarães et al. 2018) applied molecular approaches to investigate the diversity of rosy tetra clade, and they suggested that its taxonomic resolution should be better investigated as it could include cryptic species or valid species which may have been synonymized. A new species of *Hyphessobrycon* and member of

the rosy tetra clade is described from the upper Pindaré river drainage, Mearim river basin, a coastal river basin of the Eastern Amazon region, Brazil, based on both morphology and molecular data. Furthermore, a new clade, within the rosy tetra clade, is proposed based on the phylogenetic tree topology presented.

Materials and methods

Taxa sampling, specimens collection, and preservation

Individuals collected for this study were euthanized with a buffered solution of MS-222 at a concentration of 250 mg L⁻¹ for a period of 10 min or more until opercular movements completely ceased. Specimens selected for morphological analysis were fixed in formalin and left for 10 days, after which they were preserved in 70% ethanol. Molecular data were obtained from specimens that were euthanized, fixed, and preserved in absolute ethanol.

Specimens for morphological analysis are listed in type and comparative material lists. Specimens for molecular approaches are listed in Table 1. We also retrieved sequences from other species of *Hyphessobrycon* and allied species for a comparative analysis from the Barcode of Life Database (BOLD) and the National Center for Biotechnology Information (NCBI) databases (Table 1).

Morphological analysis

Measurements and counts were made according to Fink and Weitzman (1974), with exception of the scale rows below lateral line, which were counted to the insertion of pelvic-fin. Vertical scale rows between the dorsal-fin origin and lateral line do not include the scale of the median predorsal series situated just anterior to the first dorsal-fin ray. Counts of supraneurals, vertebrae, procurent caudal-fin rays, unbranched dorsal and anal-fin rays, branchiostegal rays, gill-rakers, premaxillary, maxillary, and dentary teeth were taken only from cleared and stained paratypes (C&S), prepared according to Taylor and Van Dyke (1985). The four modified vertebrae that constitute the Weberian apparatus were not included in the vertebrae counts and the fused PU1 + U1 was considered as a single element. Osteological nomenclature follows Weitzman (1962). Institutional abbreviations follow Fricke and Eschmeyer (2019), with addition of LIOP.UFAM Coleção Ictiológica do Laboratório de Ictiologia e Ordenamento Pesqueiro do Vale do Rio Madeira da Universidade Federal do Amazonas.

DNA extraction, amplification, and sequencing

DNA was extracted from fin clips using Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's protocol. Fragments of the cytochrome c oxidase subunit 1 gene (hereafter COI) from mitochondrial DNA were amplified, using the universal primers designed by Ward et al. (2005) for fish. Polymerase chain reactions (PCR) comprised a total volume of 15 µl containing 1× Polymerase buffer, 1.5 mM MgCl₂, 200 µM dNTP, 0.2 uM of each primer, 1U of Taq Polymerase (Invitrogen), 100 ng of DNA template, and ultrapure water. The PCR cycles were as follows: 2 min at 94 °C, followed by 35 cycles of 94 °C for 30s, 54 °C for 30s, and 72 °C for 1 min, and 10 min at 72 °C. Amplicons were purified using Illustra GFX PCR DNA and Gel Purification Kit (GE Healthcare Systems) and sequenced using the forward primer by an outsourced sequencing service at the University of São Paulo, using BigDye Terminator kit 3.1 Cycle Sequencing kit in ABI 3730 DNA Analyser (Applied Biosystems).

Data partition, evolution models, and alignment

The dataset included the following gene: COI (680 Base pairs, BP). Sequences were aligned using ClustalW (Chenna et al. 2003). The DNA sequences were translated into amino acids residues to test for the absence of premature stop codons or indels using the program MEGA 7 (Kumar et al. 2016). In the alignment, gaps were coded with a dash (–) and missing data with a question mark (?), but during analyses, both were treated as missing data. Measure Substitution Saturation tests were performed in DAMBE5 (Xia 2013) according to the algorithm proposed by Xia et al. (2003). The best-fit evolutionary model (GTR+G) was calculated, using the corrected Akaike Information Criterion (AICc) determined by the jModelTest 2.1.7 (Darriba et al. 2012).

Species concept, species delimitation, and diagnoses

The unified species concept is herein adopted by expressing the conceptual definition shared by all traditional species concepts, “species are (segments of) separately evolving metapopulation lineages”, disentangling operational criterion elements to delimit taxa from species concepts (de Queiroz 2005, 2007). According to this concept, species are treated as hypothetical units and could be tested by the application of distinct criteria (species delimitation methods) (de Queiroz 2005, 2007). It allows for any criteria to separately provide evidence about species limits and identities, independently from other

criteria (de Queiroz 2005, 2007). However, evidence corroborated from multiple operational criteria is considered to produce stronger support for hypotheses of lineage separation (de Queiroz 2007; Goldstein and Desalle 2010), a practice called “integrative taxonomy” (Dayrat 2005; Goldstein and Desalle 2010; Padial et al. 2010).

Five distinct and independent operational criteria for species delimitation, based on morphological and molecular data, were implemented here: Population Aggregation Analysis (Davis and Nixon 1992) (hereafter PAA); DNA barcoding, as proposed by Hebert et al. (2003a, 2003b, 2004 a, 2004b) (hereafter DBC); a tree-based method as proposed by Wiens and Penkrot (2002) (hereafter WP, following Sites and Marshall 2003); a character-based DNA barcoding as proposed by Desalle et al. (2005) (hereafter CBB); and a coalescent species delimitation method termed the Bayesian implementation of the Poisson tree processes (hereafter bPTP, following Zhang et al. 2013). All species delimitation methods here adopted, except PAA, were performed on cytochrome c oxidase subunit 1 (COI) sequences, as it is a mitochondrial gene with fast evolutionary rate, suitable for single locus species delimitation approaches (Avice 2000).

Population aggregation analysis (PAA)

The PAA (Davis and Nixon 1992) is a character-based method, in which species are delimited by unique combination of morphological character states occurring in one or more populations (Costa et al. 2014). The morphological data was based on both examined material and literature (e.g. Steindachner 1882; Meek 1904; Eigenmann, 1908; Durbin 1909; Eigenmann 1915; Ahl 1937; Fowler 1943; Géry 1960, 1961, 1964, 1977; Géry and Uj 1987; Burgess 1993; Planquette et al. 1996; Weitzman and Palmer 1997a, 1997b, 1997c, 1997d; Zarske 2008; Hein 2009; Lima et al. 2013; Zarske 2014; Carvalho and Malabarba 2015; Carvalho et al. 2017; Guimarães et al. 2018).

Traditional DNA barcoding (DBC) and Phylogenetic analysis

We used the Kimura-2-parameters model (K2P) (Kimura 1980) to estimate the pairwise genetic distances between species in MEGA 7 software (Kumar et al. 2016). We used DnaSP v. 6 (Rozas et al. 2003) to estimate the number of variable sites and haplotypes. A Bayesian inference-based phylogenetic (BI) tree was estimated in MrBayes (Huelsenbeck and Ronquist 2001) plugin in Geneious 9.0.5 to reconstruct the evolutionary relationships among terminals using General Time Reversible (GTR+G) as evolutionary

model. Bayesian tree inference was based in a chain length of 10 million, a burn-in length of 500,000 generations subsampling trees every 10,000 generations. We used a sequence of *Hyphessobrycon flammeus* Myers, 1924 as outgroup.

Wiens and Penkrot analysis (WP)

WP is based on the direct inspection of haplotype trees generated from the phylogenetic analysis having as terminals at least two individuals (haplotypes) of each focal species. In this method, the term “exclusive” is used instead of monophyletic, as the term monophyly is considered inapplicable below the species level (Wiens and Penkrot 2002). Clustered haplotypes with concordant geographic distribution forming mutual and well supported clades (exclusive lineages) are considered strong evidence for species discrimination (absence of gene flow with other lineages). When haplotypes from the same locality fail to cluster together, there is potential evidence for gene flow with other populations (Wiens and Penkrot 2002). Statistical support for clades is assessed by the posterior probability, considered as significant at values about 0.95 or higher (Alfaro and Holder 2006). When only one haplotype (specimen) from one putative population was available, the species delimitation was based on the exclusivity of the sister clade of this single haplotype, supported by significant values, allowing us to perform the test in populations with only one haplotype (Wiens and Penkrot 2002). In addition, the method allows recognition of nonexclusive lineages as species since their sister clades are exclusive and supported by significant values (Wiens and Penkrot 2002).

Character-based DNA barcoding (CBB)

The CBB is similar to the population aggregation analysis proposed by Davis and Nixon (1992), but directed to nucleotides as an alternative method for diagnosing taxa through DNA barcodes, as the original method is based on subjective cut-off distance measures to species designation (Hebert et al. 2003a, 2003b, 2004a, 2004b). This method delimits species based on a unique combination of nucleotides within a site shared by individuals of the same population or group of populations. In addition, species were diagnosed by nucleotide substitutions following Costa et al. (2014). Optimization of nucleotide substitutions among lineages of the *Hyphessobrycon micropterus* clade were obtained from the Maximum Parsimony topology, using TNT 1.5 (Goloboff and Catalano 2016). Maximum Parsimony analysis (MP) was obtained with the following parameters: traditional search, tree bisection reconnection branch swapping (TBR), 1 random seed,

setting random taxon-addition replicates to 1,000, multi-trees in effect, collapsing branches of zero length, characters equally weighted, and 10,000 trees saved per replication. MP tree branch support was given by bootstrap analysis (Felsenstein 1985), using a heuristic search with 1,000 replicates and the same settings used in the MP search, saving a maximum of 1,000 trees in each random taxon-addition replicate. The analysis was rooted on *Hyphessobrycon flammeus* Myers, 1924. Each nucleotide substitution is represented by its relative numeric position determined through sequence alignment with the complete mitochondrial genome of *Astyanax paranae* Eigenmann 1914 (KX609386.1:5503-7062 – mitochondrion complete genome), followed by the specific nucleotide substitution in parentheses. The results of this analysis are presented in Suppl. material 1: Box 1 and molecular diagnosis section.

Bayesian implementation of the poisson tree processes (bPTP)

The bPTP is a coalescent phylogeny-based species delimitation method aimed at delimiting species based on single locus molecular data (Zhang et al. 2013). An advantage of bPTP is that it does not need an ultrametric calibration like other coalescent approaches, avoiding errors and computer intensive processes (Zhang et al. 2013). The method relies on the number of substitutions between haplotypes and assumes that more molecular variability is expected between species than within a species (Zhang et al. 2013). In our analysis the dataset was reduced to include only unique haplotypes from the species of the *H. micropterus* clade. Outgroups were restricted to *Hyphessobrycon bentosi* Durbin, 1908 and *Hyphessobrycon copelandi* Durbin 1908. Sequences were aligned using ClustalW (Chenna et al. 2003). The best-fit evolutionary model (GTR+G) for the reduced dataset was calculated using the corrected Akaike Information Criterion (AICc) determined by the jModelTest 2.1.7 (Darriba et al. 2012). The input phylogenetic tree was performed in MrBayes 3.2.6 (Ronquist et al. 2012), with the following parameters: independent runs of two Markov chain Monte Carlo (MCMC) runs of four chains each for 3 million generations and sampling frequency of 1,000. The bPTP analysis was performed in the Exelixis Lab's web server <http://species.h-its.org/ptp/>, following the default parameters except for a 20% burn in.

Results

Hyphessobrycon caru sp. nov.

<http://zoobank.org/3BC35EBB-E138-4E24-A06E-DF985F015ED5>

Figures 1, 2a; Table 2

Holotype. CICCAA 02286, 22.2 mm SL, Brazil, Maranhão state, Buriticupu municipality, Buritizinho river, Pindaré river drainage, Mearim river basin, 04°22'52"S, 46°30'35"W, 24 Jan. 2017, Guimarães E. C., Brito P. S.

Paratypes. All from Brazil, Maranhão state: CICCAA 00706, 37, 15.9–25.4 mm SL; CICCAA 0709, 12 C&S, 15.1–20.6 mm SL; LIOP.UFAM 1009, 1, 16.2 mm SL collected with holotype. CICCAA00707, 3, 17.2–22.1 mm SL, Buriticupu municipality, Buritizinho river, Pindaré river drainage, Mearim river basin, 4°25'45"S, 46°29'41"W, 24 Jan. 2017, Guimarães E. C., Brito P. S. CICCAA00708, 2, 19.9–21.6 mm SL, Buriticupu municipality, Buritizinho river, Pindaré river drainage, Mearim river basin, 04°19'45"S, 46°29'46"W, 24 Jan. 2017, Guimarães E. C., Brito P. S. UFRJ11745, 1, 22.4 mm SL, Buriticupu municipality, Buritizinho river, Pindaré river drainage, Mearim river basin, 04°19'45"S, 46°29'46"W, 24 Jan. 2017, Guimarães E. C., Brito P. S.

Diagnosis (PAA). The new species *Hyphessobrycon caru* sp. nov. differs from most of its congeners, except members of the rosy tetra clade, by the presence of a dark brown or black blotch on dorsal-fin (vs absence) and absence of a midlateral stripe on the body (vs presence).

The new species differs from most of its congeners in the rosy tetra clade by possessing few irregular inconspicuous vertically arranged chromatophores in the humeral region, or sometimes a very thin and inconspicuous humeral spot (Fig. 2a) [vs inconspicuous vertically elongated humeral spot in *H. hasemani* Fowler, 1913, *H. piorskii* Guimarães, De Brito, Feitosa, Carvalho-Costa, Ottoni, 2018 (Fig. 2b); approximately rounded humeral spot in *H. erythrostigma* (Fowler, 1943), *H. jackrobertsi* Zarske, 2014, *H. minor* Durbin, 1909, *H. pando* Hein, 2009, *H. paepkei* Zarske, 2014, *H. pyrrhonotus* Burgess, 1993, *H. roseus* (Géry, 1960), *H. socolofi* Weitzman, 1977, and *H. sweglesi* (Géry, 1961) (Fig. 2c); humeral spot horizontally or posteriorly elongated in *H. epicharis* Weitzman &

Palmer, 1997, *H. khardinae* Zarske, 2008, and *H. wernerii* Géry & Uj, 1987 (Fig. 2d); conspicuous humeral spot at least on males in *H. copelandi* Durbin, 1908, *H. eques* (Steindachner, 1882), *H. haraldschultzi* Travassos, 1960, *H. micropterus* (Eigenmann, 1915), *H. megalopterus* (Eigenmann, 1915), *H. simulatus* (Géry, 1960) and *H. takasei* Géry, 1964 (Fig. 2e); and absence of humeral spot in *H. compressus* (Meek, 1904), *H. dorsalis* Zarske, 2014, *H. georgettae* Géry, 1961, *H. pulchripinnis* Ahl, 1937, and *H. rosaceus* Durbin, 1909 (Fig. 2f)].

Furthermore, the new species differs from *H. bentosi* Durbin, 1908, *H. erythrostigma*, *H. pyrrhonotus*, *H. rosaceus*, and *H. socolofi* by presenting only one tooth in the outer row of premaxillary, and this unique tooth just slightly displaced from inner row [vs two or more teeth, displaced from the inner row]; from *H. hasemani* and *H. micropterus* by the dorsal-fin spot located approximately at the middle of the fin's depth, not reaching its tip (vs spot located approximately at the middle of the fin's depth, reaching its tip in adults); from *H. hasemani* by presenting tri to unicuspid teeth in the inner row of premaxillary and dentary (vs tricuspid or pentacuspid teeth); from *H. piorskii* by having the anal-fin profile usually nearly straight (vs anal-fin profile usually falcate). In addition, *H. caru* sp. nov. is easily distinguished from *Pristella maxillaris* (Ulrey, 1894), *Moenkhausia hemigrammoides* Géry, 1965, and *Hemigrammus unilineatus* (Gill, 1858) by the absence of a black oblique stripe or band on the anterior portion of the anal-fin (Fig. 1) (vs presence).

Description. Morphometric data of holotype and paratypes are presented in Table 2. Body small (with maximum SL of 25.4 mm), compressed, moderately deep, greatest body depth slightly anterior to dorsal-fin base. Lateral body profile straight and downward directed from the end of dorsal-fin to adipose-fin, straight or slightly convex between later point and origin of dorsal most procurent caudal-fin ray. Dorsal profile of head convex from upper lip to vertical through eye; predorsal profile of body roughly straight, dorsal-fin base slightly convex, posteroventrally inclined; ventral profile of head convex from lower jaw to pelvic-fin origin. Ventral profile of body straight or slightly convex from pelvic-fin origin to anal-fin origin; straight and posterodorsally slanted along anal-fin base; and slightly concave on caudal peduncle. Jaws equal, mouth terminal, anteroventral end of dentary protruding. Maxilla reaching vertical to anterior margin of pupil.

Premaxillary teeth in two rows. Outer row with one unicuspid or tricuspid tooth, just slightly displaced from inner row; inner row with 6(5), 7(6), or 8(1) tricuspid teeth and one unicuspid tooth. Maxilla with 3(2) tricuspid teeth and two unicuspid teeth, 4(3) tricuspid teeth and two unicuspid teeth or 5(7) tricuspid teeth. Dentary with five (10) or six (1) larger tricuspid teeth followed by one smaller tricuspid teeth 5(2), 6(2), 7(3), and 8(5) smaller unicuspid teeth (Fig. 3).

Scales cycloid, three to eight radii strongly marked, circuli well-marked anteriorly, weakly marked posteriorly; lateral line incompletely pored, with 5(1), 6(2), 7(24), 8(14), or 9(4) perforated scales. Longitudinal scales series including lateral-line scales 31(1), 32(7), 33(14), 34(13), 35(3), or 36(7). Longitudinal scales rows between dorsal-fin origin and lateral line 5(3), 6(32), or 7(10). Horizontal scale rows between lateral line and pelvic-fin origin 4(43) or 5(2). Scales in median series between tip of supraoccipital spine and dorsal-fin origin 10(9), 11(12), 12(21), or 13(3). Circumpeduncular scales 11(6), 12(35), 13(2), or 14(2).

Dorsal-fin origin at midbody. Base of last dorsal-fin ray at vertical through first third of anal-fin. Dorsal-fin rays ii + 9(48), iii + 9(5), ii + 10(4). First dorsal-fin pterygiophore main body located behind neural spine of 4th vertebrae. Adipose-fin present. Anal-fin origin aligned with vertical line through middle of dorsal-fin, between 6th and 8th dorsal-fin rays base. Anteriormost anal-fin pterygiophore inserting posterior to haemal spine of 11th vertebrae. First anal-fin ray in vertical through the middle of dorsal-fin (with about 7th or 8th ray base). Anal-fin iii + 22(10) or iii + 23(47); anal-fin origin aligned with vertical line through middle of dorsal-fin (between base of 6th and 8th dorsal-fin rays); Anal-fin profile nearly straight; Anal-fin rays with a sexually dimorphic pattern, which is absent in females, described below. Pectoral-fin rays 12(57) total rays. Tip of pectoral-fin surpassing pelvic-fin base. Pelvic-fin rays 8(57) total rays, surpassing anal-fin origin. Pelvic-fin rays with a sexually dimorphic pattern, which are absent in females, described below. Caudal-fin forked, upper and lower lobes similar in size. Principal caudal-fin rays 11+10(50) or 10+9(7); dorsal procurrent rays 8(2), 9(8) or 11(2) and ventral procurrent rays 7(4) or 8(8).

Branchiostegal rays 4(12). First gill arch with 1(11), 2(1) hypobranchial, 11(1), 12(10), or 13(1) ceratobranchial, 1(12) on cartilage between ceratobranchial and epibranchial,

and 5(1) or 6(11) epibranchial gill-rakers. Supraneurals 3(2), 4(9), or 5(1). Total vertebrae 28(2) or 29(10).

Colour in alcohol. Ground coloration light yellowish brown. Humeral region with few irregular inconspicuous vertically arranged chromatophores, sometimes very thin and inconspicuous humeral spot. Flank with chromatophores homogeneously scattered, more concentrated on posterior region to humeral spot, posterior region of dorsal-fin base origin and below mid-portion of trunk, between anal-fin origin and caudal peduncle. Ventral region lacking dark-brown chromatophores. Dark-brown chromatophores present on head and more concentrated on dorsal portion, becoming sparser on cheek and preopercular regions.

Dorsal-fin ground coloration hyaline, with conspicuous black or dark-brown spot located on anterior portion of fin, reaching about 6th ray, approximately between one-half to two-thirds of fin depth. Anal and caudal-fins hyaline. Caudal-fin with a darker, usually dark brown, posterior margin and on its base. Adipose-fin hyaline to light brown, with dark-brown or black chromatophores more concentrated on its dorsal portion, depending on the specimen preservation state. Pectoral and pelvic-fins hyaline; pelvic-fin with variable amounts of dark-brown pigmentation remaining depending on the specimen preservation state.

Sexual dimorphism. Mature males with small bone hooks on anal and pelvic-fin rays. Bone hooks absent on females. Anal-fin presenting bone hooks from 3rd, 4th, or 5th rays to the last ray. Number of hooks variable, increasing from the first to the last rays. Pelvic-fin presenting 2nd, 3rd, 4th, or 5th rays with 5, 6, or 7 smaller hooks.

Etymology. The specific epithet honors the term “*Caru*”. Caru is the name of an area (about 70.000 ha) inhabited by Brazilian native tribes from the ethnicities Guajá and Guajajara. People from this area use the Tupi language and have suffered consequences of European colonization and are under threat due to the pressure for exploration of the protected territory.

Geographic distribution. *Hyphessobrycon caru* sp. nov. has a restricted geographic distribution, being known only from the upper Pindaré river drainage, Mearim river basin, in the state of Maranhão, northeastern Brazil (Fig. 4). This species was never collected in

the lower portions of this river drainage during 8 years of field trips conducted by EG and PB, including about 15 expeditions.

Molecular diagnosis (CBB). *Hyphessobrycon caru* sp. nov. belongs to the *H. micropterus* clade possessing 20 synapomorphic nucleotide substitutions: COI 73 (C→T), COI 88 (T→C), COI 217 (C→T), COI 274 (C→T), COI 298(C→T), COI 334 (C→G), COI 338 (T→C), COI 370 (A→G), COI 418 (A→G), COI 433 (C→T), COI 439 (C→A), COI 457 (A→G), COI 469 (T→C), COI 478 (A→T), COI 559 (A→G), COI 562 (T→A), COI 592(A→G), COI 631 (A→T), COI 655 (A→C), COI 673 (A→C). It shares nine synapomorphic nucleotide substitutions with *H. piorskii*, which separate them from *H. simulatus* and *H. micropterus*: COI 181 (A→C), COI 208 (A→G), COI 245 (C→T), COI 325 (T→C), COI 349 (T→C), COI 436 (A→T), COI 472 (A→G), COI 538 (C→T), COI 556 (T→C). In addition, it has six unique nucleotide substitutions within the *H. micropterus* clade: COI 148 (C→T), COI 154 (C→T), COI 175 (T→C), COI 364 (G→A), COI 487 (T→C), COI 517 (A→G) (Fig. 5; Suppl. material 1: Box 1).

DBC. COI sequences support the existence of a new species of *Hyphessobrycon* inhabiting the Pindaré river basin in Maranhão state. After trimming, the final alignment yielded 680 base pairs with 159 polymorphic sites and 26 haplotypes. Average genetic distances were 18.3%, with the highest values between *H. epicharis* and *H. erythrostigma* (23.4%), while the lowest value (0.7%) was between *H. pyrrhonotus* and *H. erythrostigma* (Table 3). *Hyphessobrycon caru* sp. nov. is divergent on average 17.0% from the other taxa, with a minimum distance of 3.6% to *H. piorskii* and a maximum of 21.8% to *Pristella maxillaris* (Table 3).

WP and CBB. Both phylogenetic analysis based on BI and MP supported a clade comprising *H. caru* sp. nov., *H. micropterus*, *H. piorskii*, and *H. simulatus*, hereafter termed *Hyphessobrycon micropterus* clade, with maximum posterior probability value and 99% bootstrap value in BI and MP, respectively. *Hyphessobrycon caru* sp. nov. formed a single exclusive lineage with maximum posterior probability value (posterior probability = 1) and 99% bootstrap value in BI and MP, respectively.

These species delimitation analysis (WP and CBB) have identical results, delimitating four species within the *Hyphessobrycon micropterus* clade: *H. caru* sp. nov., *H. micropterus*, *H. piorskii*, and *H. simulatus* (Fig. 5a, b). The nucleotide substitutions

supporting these four lineages within the *H. micropterus* clade, and the nucleotide substitutions supporting this clade are presented in Figure 5b and Suppl. material 1: Box 1. The combination of nucleotide substitutions diagnosing *H. caru* sp. nov. are presented in the molecular diagnosis section.

bPTP. This species delimitation analysis also indicates four lineages (species) within the *Hyphessobrycon micropterus* clade: *H. caru* sp.n., *H. micropterus*, *H. piorskii*, and *H. simulatus* (Fig. 6). This outcome was similar to the aforementioned results. The species included as outgroups (*H. bentosi* and *H. copelandi*) were also supported as independent lineages.

Discussion

Currently molecular techniques are frequently useful for solve species complexes and discover cryptic species (e.g. Bickford et al. 2006; Costa and Amorim 2011; Pereira et al. 2011; Adams et al. 2014; Costa-Silva 2015; Costa et al. 2012, 2014, 2017; Amorim 2018; Guimarães et al. 2018; Ottoni et al. 2019) and could be an excellent complement for traditional taxonomy (Kekkonen and Hebert 2014). DNA barcoding has demonstrated to be very efficient for delimiting species of *Hyphessobrycon*, mainly in groups with little morphological variation (i.e., cryptic species) (see Castro-Paz et al. 2014; Guimarães et al. 2018), preferably when applied together with other species delimitation methods, such as PAA, DBC, CBB, bPTP, and WP in an integrative taxonomy perspective (Guimarães et al. 2018). The recognition of different genetic patterns and lineages in groups with very similar morphology has been a common pattern in the tree of eukaryotic life. This is observed particularly often in species-rich genera, such as in several Neotropical fishes (e.g. Pereira et al. 2011; Roxo et al. 2012; Castro-Paz et al. 2014; Melo et al. 2014, 2016a; Benzaquem et al. 2015; Benine et al. 2015; Ottoni et al. 2019). DNA techniques can help to uncover morphological hidden diversity (Bickford et al. 2006; Adams et al. 2014), delimiting a putative population or group of populations as an independent lineage (species), and, subsequently, through a more meticulous analysis of morphological features, morphological differences between cryptic species can be found.

The large number of the described *Hyphessobrycon* species (about 160 spp.), with new species described every year, reveal an astonishing diversity within the genus. During the past 10 years, about 50 new species have been described (Fricke et al. 2019). However, historically *Hyphessobrycon* species have been described only on the basis of

morphological features, including differences in the pigmentation patterns and teeth numbers and morphology, using few individuals per species (e.g. Steindachner 1882; Eigenmann 1915; Zarske 2008, 2014; Bragança et al. 2015). Recently, DNA barcoding in characoid fishes has been used to discriminate species, identify new ones, and reveal that it is not always possible to differentiate species based solely on their morphology (Ornelas-Garcia et al. 2008; Pereira et al. 2011; Castro-Paz et al. 2014; Melo et al. 2014, 2016a; Benine et al. 2015).

Our results suggest a cryptic speciation in the rosy tetra clade, more specifically in a new clade here defined, the *Hyphessobrycon micropterus* clade, including *H. caru* sp. nov., *H. micropterus*, *H. piorskii*, and *H. simulatus*, so far only known from the Pindaré, Itapecuru, Munim, Preguiças, and São Francisco river drainages of Brazil and the coastal river basins of French Guiana and Suriname (Guimarães et al. 2018; Brito et al. 2019; Fricke et al. 2019; this study). The clade proposed here is supported by high node support values (maximum posterior probability value and 99% of bootstrap value in BI and MP, respectively). In addition, this clade was corroborated by 20 synapomorphic nucleotide substitutions (Fig. 5; Suppl. material 1: Box 1).

Hyphessobrycon caru sp. nov. is herein described within the *Hyphessobrycon micropterus* clade based on five different and independent methods of species delimitation (PAA, DBC, WP, CBB and bPTP), characterized by different criteria and assumptions. *Hyphessobrycon caru* sp. nov. is distinguished from all its congeners by a combination of unambiguous morphological character states [see Diagnosis (PAA)]. In our Bayesian phylogenetic analysis (Fig. 5A), haplotypes of *H. caru* sp. nov. formed a single exclusive clade with maximum posterior probability value (posterior probability = 1) (WP). Furthermore, the COI average genetic distance of *H. caru* sp. nov. when compared with the other taxa herein analyzed was 19.6% and its minimum COI genetic distance was 3.6% to *H. piorskii* (DBC). Considering this value, the threshold of *H. caru* sp. nov. would be greater than that inferred by delimitations among Neotropical fish species (2% according to Pereira et al. 2011). Moreover, *H. caru* sp. nov. was also molecularly diagnosed by six synapomorphic nucleotide substitutions (Fig. 5b; Suppl. material 1: Box 1), as well as, by a combination of other nucleotide substitutions (see CBB - molecular diagnosis), and corroborated by a bPTP analysis. Thus, it makes the hypothesis of this new species stronger from an integrative taxonomy perspective (see

Dayrat 2005; de Queiroz 2007; Goldstein and Desalle 2010; Padial et al. 2010). Therefore, we recommend the use of integrative taxonomy for future taxonomic revisions and species descriptions when dealing with species complexes and groups containing possible cryptic species.

Comparative material

Hyphessobrycon amandae: UFRJ 1557, 5 spcms, Jussara municipality, Goiás state, Brazil. ***H. bentosi***: MCZ 20842, 1 spcm (Syntype), Óbidos municipality, Pará state, Brazil. ***H. bifasciatus***: UFRJ 0068, 6 spcms, Marataízes and Guarapari municipality, Espírito Santo state, Brazil. ***H. compressus***: BMNH 1905.12.6.4-5, 2 spcms (Paratypes), Oaxaca state, México. ***H. copelandi***: CAS 42683, 1 spcm (Syntype); MCZ 20771, 1 spcm (Syntype), Tabatinga municipality, Amazonas state, Brazil. ***H. eques***: CICCAA 00715, 4 spcms (C&S); CICCAA 00710, 51 spcms, Tombos municipality Carangola river, Minas Gerais state, Brazil. ***H. erythrostigma***: ANSP 70208, 1 spcm (Holotype), Peru and Brazil. ***H. epicharis***: FMNH100609, 1 spcm (Paratype), Baria river, Amazonas, Venezuela. ***H. haraldschultzi***: CICCAA 00873, 20 spcms, Ilha do Bananal municipality, Javaés river, Tocantins state, Brazil. ***H. hasemani***: ANSP 39230, 1 spcm (Holotype), Guajaramirim municipality, Madeira river, Rondônia state, Brazil. ***H. micropterus***: FMNHH 57916, 1 spcm (Holotype), São Francisco river at Lagoa de Porto, Minas Gerais state, Brazil. ***H. piorskii***: CICCAA 00695, 1 spcm (Holotype); CICCAA 00430, 15 spcms (Paratype); CICCAA 00431, 21 spcms (Para Paratype); CICCAA 00696, 15 spcms (Paratype); CICCAA 00697, 16 spcms (C&S) (Paratype); CICCAA 00698, 6 spcms, 1 spcm (C&S) (Paratype); CICCAA 00750, 9 spcms (Paratype); CICCAA01654, 1 spcm (Paratype); CPUFMA 171664, 15 spcms (Paratype); UFRJ 11553, 6 spcms (Paratype), stream at the Anapurus municipality, Munim river, Maranhão state, Brazil. CICCAA 00089, 1 spcm (C&S) (Paratype); CICCAA 00881, 1 spcm (Paratype); CICCAA 01563, 1 spcm (Paratype); stream at Mata de Itamacaoca, Chapadinha municipality, Munim river, Maranhão state, Brazil. CICCAA 01382, 5 spcms (Paratype); CICCAA 02008, 12 (C&S) spcms (Paratype), stream at Mata Fome, Barreirinhas municipality, Preguiças river, Maranhão state, Brazil. ***H. pyrrhonotus***: MZUSP 45714, 1 spcm (Holotype), Ereré river, Brazil. ***H. rosaceus***: FMNH 52791, 1 spcm (Holotype), Gluck Island, Essequibo River, Guyana. ***H. werneri***: MZUSP 42365, 1 spcm (Holotype), Santa Maria do Pará and São Miguel de Guamá municipality, Guamá river, Pará state, Brazil. CICCAA 00751, 1 spcm,

Paragominas municipality, Candiru river, Pará state, Brazil. *H. socolofi*: MZUSP 13181, 1 spcm (Holotype), Barcelos municipality, Negro river, Amazonas state, Brazil.

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Table 1. List of species, specimens and their respective catalogue numbers, Region/State/Country, coordinates and BOLD Systems and GenBank sequence accession numbers. Sequences available in the current study are underlined.

N°	Species	Catalogue number	Region/State/Country	Coordinates	Accession no.
1	<i>Hyphessobrycon erythrostigma</i>	INPA 37681-HERY1	Tabatinga/Amazonas/Brazil	4°12'15.98"S 69°55'14.16"W	HYP076-13
2	<i>Hyphessobrycon erythrostigma</i>	INPA 37681-	Tabatinga/Amazonas/Brazil	4°12'15.98"S 69°55'14.16"W	HYP077-13
3	<i>Hyphessobrycon erythrostigma</i>	INPA 37681-HERY2	Tabatinga/Amazonas/Brazil	4°12'15.98"S 69°55'14.16"W	HYP078-13
4	<i>Hyphessobrycon erythrostigma</i>	INPA 37681-HERY3	Tabatinga/Amazonas/Brazil	4°12'15.98"S 69°55'14.16"W	HYP079-13
5	<i>Hyphessobrycon pyrrhonotus</i>	INPA 37672-TRO10	Santa Isabel do Rio	0°33'6.98"S 64°48'6.12"W	HYP040-13
6	<i>Hyphessobrycon pyrrhonotus</i>	INPA 37672-TRO11	Santa Isabel do Rio	0°33'6.98"S 64°48'6.12"W	HYP041-13
7	<i>Hyphessobrycon pyrrhonotus</i>	-	Barcelos/Amazonas/Brazil	-	HYP157-13
8	<i>Hyphessobrycon pyrrhonotus</i>	-	Barcelos/Amazonas/Brazil	-	HYP158-13
9	<i>Hyphessobrycon socolofi</i>	INPA_39530-6152	Barcelos/Amazonas/Brazil	-	HYP131-13
10	<i>Hyphessobrycon socolofi</i>	INPA_39530-6155	Barcelos/Amazonas/Brazil	-	HYP134-13
11	<i>Hyphessobrycon socolofi</i>	INPA_39530-6178	Barcelos/Amazonas/Brazil	-	HYP135-13
12	<i>Hyphessobrycon socolofi</i>	INPA 39530-BCR8	Barcelos/Amazonas/Brazil	-	HYP148-13
13	<i>Hyphessobrycon copelandi</i>	INPA_37683-TU1	Tabatinga/Amazonas/Brazil	4°12'15.98"S 69°55'14.16"W	HYP094-13
14	<i>Hyphessobrycon copelandi</i>	INPA_37683-TU2	Tabatinga/Amazonas/Brazil	4°12'15.98"S 69°55'14.16"W	HYP095-13
15	<i>Hyphessobrycon copelandi</i>	INPA_37683-TU3	Tabatinga/Amazonas/Brazil	4°12'15.98"S 69°55'14.16"W	HYP096-13
16	<i>Hyphessobrycon eques</i>	INPA_37678-IC2	Santarém/Pará/Brazil	2°28'20.60"S 54°39'23.40"W	HYP070-13
17	<i>Hyphessobrycon eques</i>	INPA_37679-PE1	Macapá/Amapá/Brazil	0°17'24.40"N 50°53'16.44"W	HYP071-13
18	<i>Hyphessobrycon eques</i>	INPA_37680-AL1	Parintins/Amazonas/Brazil	2°39'53.21"S 56° 5'51.00"W	HYP072-13
19	<i>Hyphessobrycon eques</i>	-	Bonito/Mato Grosso do Sul/Brazil	-	DSMIS077-09
20	<i>Hyphessobrycon epicharis</i>	INPA_37665-JUF1	São Gabriel da	0°22'11.60"N 67°18'49.32"W	HYP002-13
21	<i>Hyphessobrycon epicharis</i>	INPA_37665-JUF3	São Gabriel da	0°22'11.60"N 67°18'49.32"W	HYP004-13
22	<i>Hyphessobrycon epicharis</i>	INPA_37665-JUF4	São Gabriel da	0°22'11.60"N 67°18'49.32"W	HYP005-13
23	<i>Hyphessobrycon epicharis</i>	INPA_37665-JUF8	São Gabriel da	0°22'11.60"N 67°18'49.32"W	HYP006-13
24	<i>Hyphessobrycon compressus</i>	CINV-NEC7411	Flores Magon/Campeche/México	18°47'20.40"N 89° 9'43.20"W	FYPM054-10
25	<i>Hyphessobrycon compressus</i>	-	Hattie ville/Belize/Belize	17°27'29.52"N 88°23'34.44"W	MXV765-15

26	<i>Hyphessobrycon compressus</i>	-	Hatie ville/Belize/Belize	17°27'29.52"N 88°23'34.44"W	MXV766-15
27	<i>Hyphessobrycon compressus</i>	-	Hatie ville/Belize/Belize	17°27'29.52"N 88°23'34.44"W	MXV767-15
28	<i>Hyphessobrycon bentosi</i>	INPA_37684-5939	Barcelos/Amazonas/Brazil	0°41'48.01"S 63°14'12.84"W	HYP097-13
29	<i>Hyphessobrycon bentosi</i>	INPA_37684-5940	Barcelos/Amazonas/Brazil	0°41'48.01"S 63°14'12.84"W	HYP098-13
30	<i>Hyphessobrycon bentosi</i>	INPA_39527-BA1	-	-	HYP116-13
31	<i>Hyphessobrycon bentosi</i>	INPA_39527-BA2	-	-	HYP117-13
32	<i>Hyphessobrycon bentosi</i>	CICCAA02349	Santarém/Pará/Brazil	2°27'44.60"S 54°39'49.93"W	MK240339
33	<i>Hyphessobrycon bentosi</i>	CICCAA02350	Santarém/Pará/Brazil	2°27'44.60"S 54°39'49.93"W	MK240340
34	<i>Hyphessobrycon bentosi</i>	CICCAA02351	Santarém/Pará/Brazil	2°27'44.60"S 54°39'49.93"W	MK240341
35	<i>Hyphessobrycon simulatus</i>	MHNG 2743.087	Pisiemoengo/Commewijne/Suriname	5°18'56.02"N 54°50'0.96"W	GBOL761-15
36	<i>Hyphessobrycon simulatus</i>	MHNG 2743.087	Pisiemoengo/Commewijne/Suriname	5°18'56.02"N 54°50'0.96"W	GBOL762-15
37	<i>Hyphessobrycon simulatus</i>	-	Sinnamary/Cayenne/French Guiana	5°25'20.32"N 53° 3'30.96"W	GBOL1771-17
38	<i>Hyphessobrycon simulatus</i>	MHNG 2735.007	Sinnamary/Cayenne/French Guiana	5°21'28.69"N 53° 0'12.60"W	GBOL3296-18
39	<i>Hyphessobrycon simulatus</i>	MHNG 2757.080	Kourou/Cayenne/French Guiana	5° 9'2.05"N 52°42'0.36"W	GBOL3298-18
40	<i>Hyphessobrycon simulatus</i>	MHNG 2759.026	Kaw/Cayenne/French Guiana	4°29'52.19"N 52° 3'9.00"W	GBOL3300-18
41	<i>Hyphessobrycon simulatus</i>	MHNG 2759.026	Kaw/Cayenne/French Guiana	4°29'52.19"N 52° 3'9.00"W	GBOL3301-18
42	<i>Hyphessobrycon simulatus</i>	MHNG 2759.035	Régina/ Cayenne/French Guiana	4° 8'28.10"N 52° 5'11.40"W	GBOL3302-18
43	<i>Hyphessobrycon cf. sweglesi</i>	INPA_37668-JAR3	São Gabriel da	0°20'10.00"S 65°18'32.04"W	HYP026-13
44	<i>Hyphessobrycon cf. sweglesi</i>	INPA_37668-JAR4	São Gabriel da	0°20'10.00"S 65°18'32.04"W	HYP027-13
45	<i>Hyphessobrycon cf. sweglesi</i>	INPA_37668-JAR5	São Gabriel da	0°20'10.00"S 65°18'32.04"W	HYP028-13
46	<i>Hyphessobrycon cf. sweglesi</i>	INPA_37668-JAR7	São Gabriel da	0°20'10.00"S 65°18'32.04"W	HYP030-13
47	<i>Hyphessobrycon micropterus</i>	-	Várzea da Palma/Minas Gerais/Brazil	17°36'7.20"S 44°40'1.20"W	BSB287-10
48	<i>Hyphessobrycon micropterus</i>	-	Várzea da Palma/Minas Gerais/Brazil	17°36'7.20"S 44°40'1.20"W	BSB288-10
49	<i>Hyphessobrycon micropterus</i>	-	Várzea da Palma/Minas Gerais/Brazil	17°36'7.20"S 44°40'1.20"W	BSB289-10
50	<i>Hyphessobrycon micropterus</i>	-	Várzea da Palma/Minas Gerais/Brazil	17°36'7.20"S 44°40'1.20"W	BSB290-10
51	<i>Hyphessobrycon pionskii</i>	CICCAA00725-1	Chapadinha/Maranhão/Brazil	03°40'14"S 43° 07'10"W	MF765796
52	<i>Hyphessobrycon pionskii</i>	CICCAA00726-1	Chapadinha/Maranhão/Brazil	03°40'14"S 43° 07'10"W	MF765797
53	<i>Hyphessobrycon pionskii</i>	CICCAA01650-1	Barreirinhas/Maranhão/Brazil	02°39'47"S 42° 48'16"W	MG791915

54	<i>Hyphessobrycon piorskii</i>	CICCAA01651-1	Barreirinhas/Maranhão/Brazil	02°39'47"S 42° 48'16"W	MG791914
55	<i>Hyphessobrycon piorskii</i>	CICCAA02164-1	Codó/Maranhão/Brazil	04°31'48"S 043°56'09"W	<u>MK240337</u>
56	<i>Hyphessobrycon piorskii</i>	CICCAA02164-4	Codó/Maranhão/Brazil	04°31'48"S 043°56'09"W	<u>MK240338</u>
57	<i>Hyphessobrycon caru</i>	CICCAA00748-1	Buriticupu/Maranhão/Brazil	04°22'52"S 46°30'35"W	<u>MH338230</u>
58	<i>Hyphessobrycon caru</i>	CICCAA00749-1	Buriticupu/Maranhão/Brazil	04°22'52"S 46°30'35"W	<u>MH338231</u>
59	<i>Hyphessobrycon caru</i>	CICCAA02300-1	Buriticupu/Maranhão/Brazil	04°22'52"S 46°30'35"W	<u>MH338232</u>
60	<i>Hyphessobrycon caru</i>	CICCAA02301-1	Buriticupu/Maranhão/Brazil	04°22'52"S 46°30'35"W	<u>MH338233</u>
61	<i>Pristella maxillaris</i>	-	-	-	KU568982.1
62	<i>Pristella maxillaris</i>	-	-	-	KU568981.1
63	<i>Pristella maxillaris</i>	-	Marlborough/Pomeroon-	7°28'59.99"N 58°37'59.88"W	TZGAA025-06
64	<i>Pristella maxillaris</i>	-	Santa Cruz/Barima-Waini/Guyana	7°40'0.01"N 59°13'59.88"W	TZGAA178-06
65	<i>Moenkhausia hemigrammoides</i>	INPA38532-PR1	Guyana	3°54'27.29"N 59°21'36.36"W	HYP101-13
66	<i>Moenkhausia hemigrammoides</i>	INPA_38532-PR2	Guyana	3°54'27.29"N 59°21'36.36"W	HYP102-13
67	<i>Moenkhausia hemigrammoides</i>	INPA_38532-PR3	Guyana	3°54'27.29"N 59°21'36.36"W	HYP103-13
68	<i>Hyphessobrycon panamensis</i>	STRI-05303	Cocle/Panama	8°48'16.70"N 80°31'59.88"W	BSFFA760-07
69	<i>Hyphessobrycon flammeus</i>	LBPV-40464	Biritiba-Mirim/São Paulo/Brazil	23°34'35.04"S 45°58'28.20"W	FUPR988-09

Table 2. Morphometric data (N=45) for the holotype and paratypes of *Hyphessobrycon caru* sp. n. SD: Standard deviation.

	Holotype	Paratypes	Mean	SD
Standard length	22.2	14.8-25.4	18.9	-
Percentages of standard length				
Depth at dorsal-fin origin (body depth)	37.3	33.1 - 38.5	35.2	1.1
Snout to dorsal-fin origin	53.7	49.4 - 55	51.7	1.2
Snout to pectoral-fin origin	29.5	28.2 - 32.3	29.9	1.0
Snout to pelvic-fin origin	46.0	43.6 - 48.8	45.6	1.0
Snout to anal-fin origin	62.5	58.5 - 64.0	61.0	1.3
Caudal peduncle depth	12.3	8.5 - 12.3	10.3	0.8
Caudal peduncle length	11.7	9.5 - 12.7	11.2	0.8
Pectoral-fin length	23.2	16.5 - 23.7	19.6	1.9
Pelvic-fin length	20.6	14.1 - 20.5	17.4	1.4
Dorsal-fin base length	15.2	12.9 - 15.7	14.3	0.8
Dorsal-fin height	32.2	27.9 - 34.1	30.8	1.5
Anal-fin base length	32.4	26.4 - 32.7	29.6	1.3
Eye to dorsal-fin origin	37.5	34.4 - 38.8	37.3	0.9
Dorsal-fin origin to caudal-fin base	55.1	50.6 - 56.1	53.4	1.1
Head length	29.8	27.4 - 31.1	29.3	1.0
Percentages of head length				
Horizontal eye diameter	39.2	35.4 - 43.6	39.2	1.7
Snout length	24.4	17.3 - 24.3	21.5	1.8
Least interorbital width	29.1	22.4 - 30.7	27.2	1.8
Upper jaw length	37.8	33.1 - 42.5	37.4	2.1

Table 3. Kimura-2 parameters pairwise genetic distances among species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	<i>H. erythrostigma</i>															
2	<i>H. pyrrhonotus</i>	0.007														
3	<i>H. socolofi</i>	0.037	0.035													
4	<i>H. simulatus</i>	0.192	0.194	0.179												
5	<i>H. micropterus</i>	0.205	0.202	0.198	0.041											
6	<i>H. piorskii</i>	0.188	0.185	0.178	0.063	0.064										
7	<i>H. caru</i>	0.206	0.210	0.203	0.062	0.057	0.036									
8	<i>H. eques</i>	0.175	0.178	0.163	0.154	0.157	0.160	0.160								
9	<i>H. copelandi</i>	0.192	0.189	0.186	0.170	0.176	0.158	0.168	0.102							
10	<i>H. epicharis</i>	0.234	0.230	0.220	0.170	0.175	0.189	0.190	0.187	0.197						
11	<i>H. bentosi</i>	0.106	0.103	0.112	0.197	0.194	0.205	0.204	0.195	0.220	0.219					
12	<i>H. cf. sweglesi</i>	0.210	0.207	0.197	0.182	0.187	0.201	0.209	0.181	0.197	0.030	0.222				
13	<i>P. maxillaris</i>	0.225	0.232	0.206	0.201	0.211	0.220	0.218	0.194	0.213	0.180	0.202	0.183			
14	<i>M. hemigrammoides</i>	0.219	0.219	0.209	0.179	0.185	0.178	0.179	0.203	0.211	0.196	0.221	0.199	0.166		
15	<i>H. compressus</i>	0.214	0.218	0.215	0.202	0.208	0.198	0.208	0.198	0.201	0.212	0.212	0.212	0.215		
16	<i>H. panamensis</i>	0.210	0.213	0.209	0.202	0.199	0.179	0.187	0.215	0.229	0.221	0.213	0.218	0.208	0.145	
17	<i>H. flammeus</i>	0.201	0.201	0.198	0.169	0.174	0.186	0.204	0.203	0.205	0.192	0.200	0.189	0.200	0.170	0.196

Figure 1. *Hyphessobrycon caru* sp. n. – CICCAA 02286, holotype, 22.2 mm SL; Brazil: Maranhão State: Buritizinho River, Pindaré river drainage, Mearim River basin.



Figure 2. Humeral blotches of: (a) *Hyphessobrycon caru*, holotype, CICCAA 02286. (b) *H. piorskii*, holotype, CICCAA00695. *H. pyrrhonotus*, holotype, MZUSP 45714. *H. werner*i, holotype, MZUSP 42365. *H. compressus*, paratype, MHNG 2181.076.

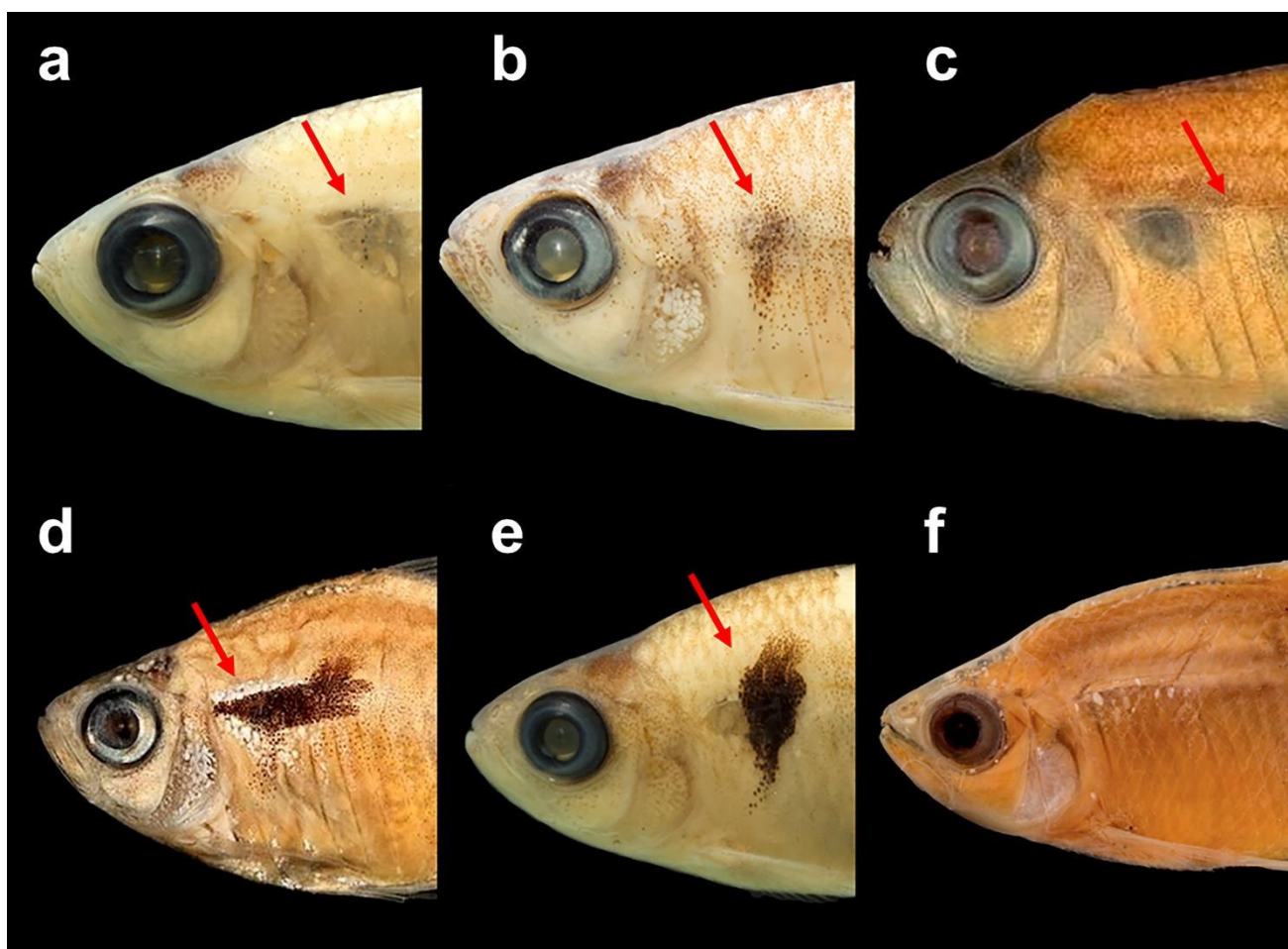


Figure 3. *Hyphessobrycon caru* sp. n. CICCAA 00697, paratype, 19.3 mm SL; jaw suspensory: **a.** Premaxillary. **b.** Maxilla. **c.** Dentary.

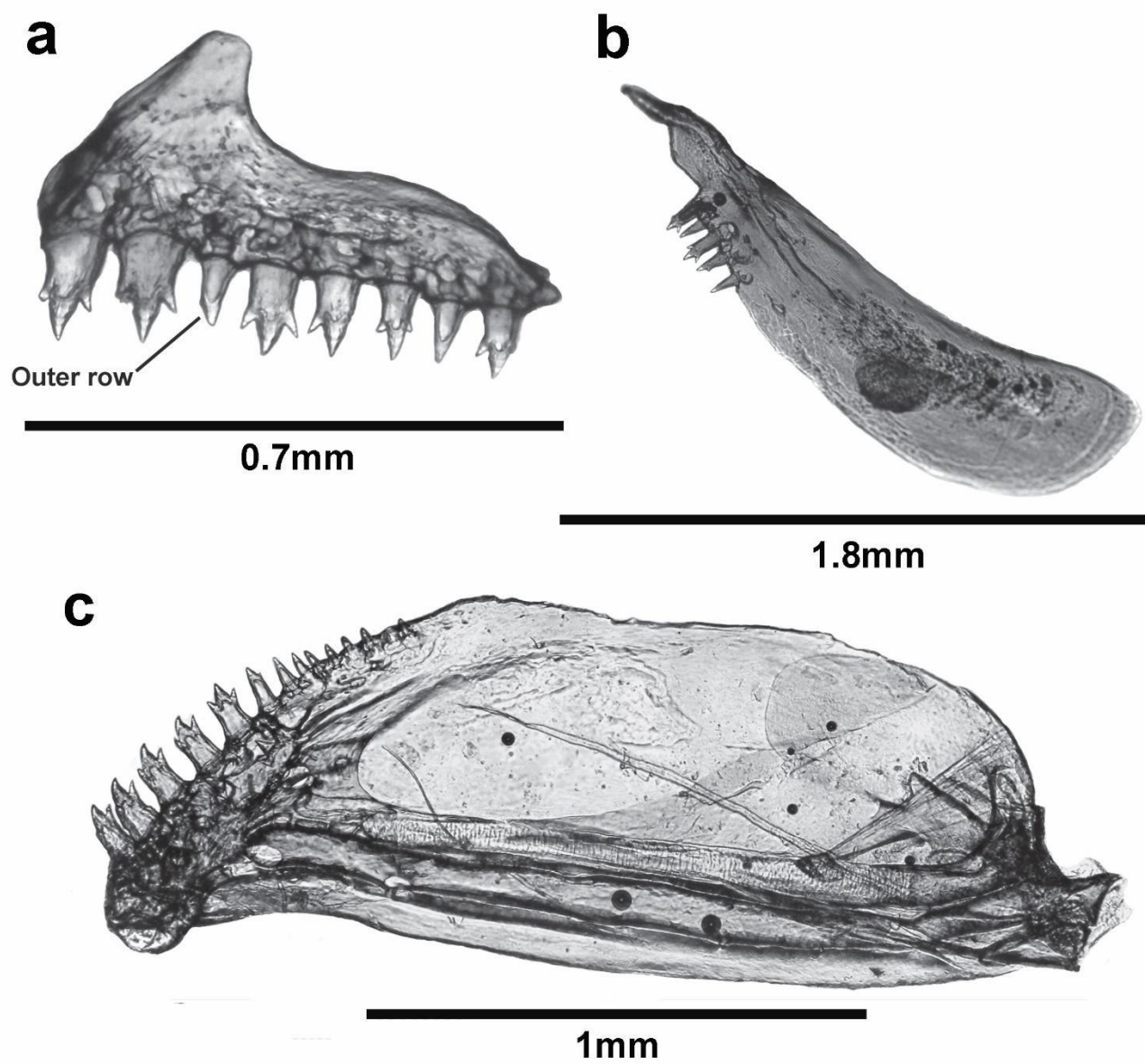


Figure 4. Geographical distribution of *Hyphessobrycon caru* sp. n. and *Hyphessobrycon piorskii*, red star denotes Holotype and blue circle denote paratypes of *Hyphessobrycon caru* sp. n., and white star denote Holotype and the black circle denotes the known distribution of *Hyphessobrycon piorskii*.

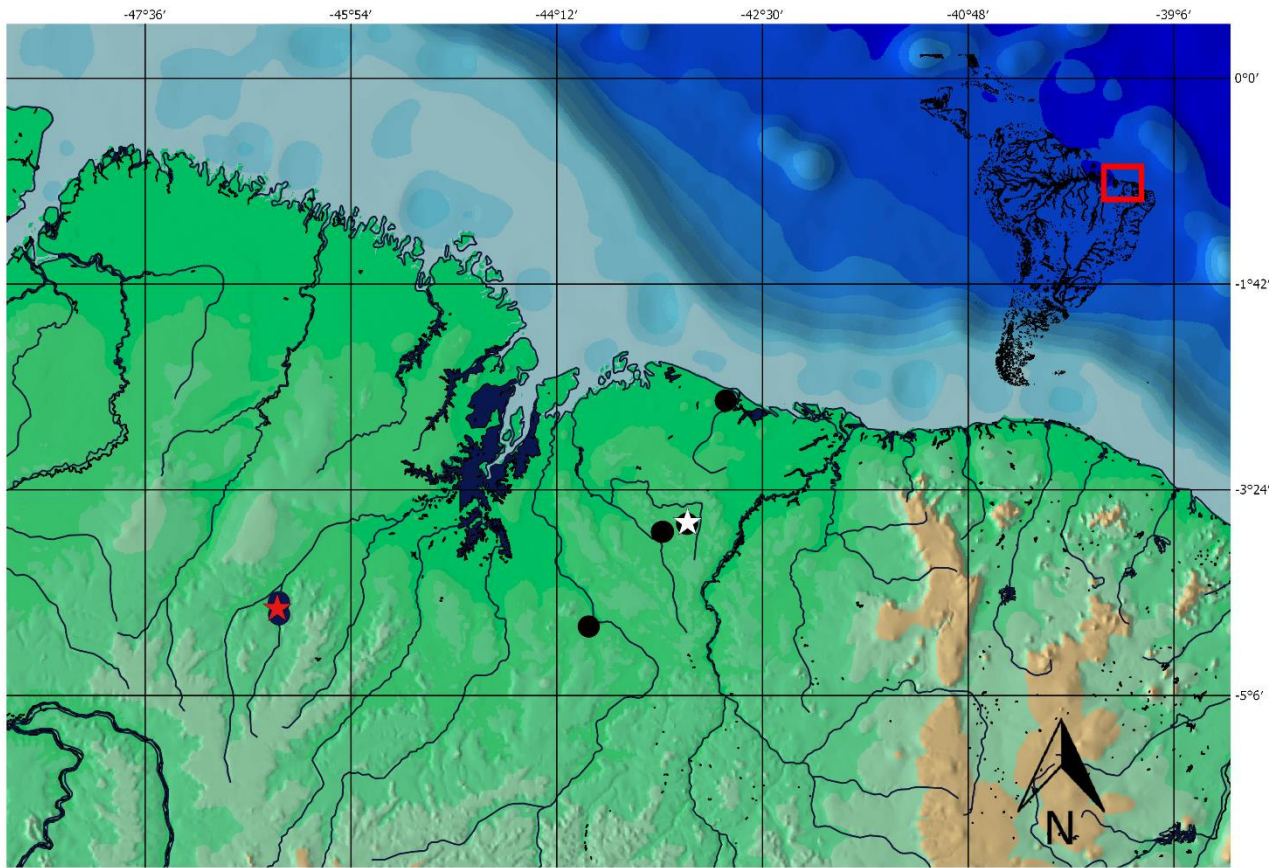


Figure 5. a. Phylogenetic tree based on Bayesian Inference (BI). Numbers above branches are posterior probability values. Posterior probability value supporting the *Hyphessobrycon micropterus* clade is indicated in green (haplotypes marked with a green bar); posterior probability value supporting the *H. caru* sp. n. lineage under WP method is indicated in red (haplotypes marked with a red bar); and the other species (lineages) under WP method, within this clade, are indicated in black. **b.** Strict consensus phylogenetic tree based on Maximum Parsimony (MP), obtained from the 38 most parsimonious trees, in which 587 characters were constant, 20 variable but parsimony-uninformative, and 248 parsimony-informative, (total length 833, consistency index 0.489, retention index 0.901). The image is focusing on the *Hyphessobrycon micropterus* clade. Numbers above branch are bootstrap value and letters below branch corresponds to nucleotide substitutions listed in Box 1 corresponding to the CBB method. Green circle indicating *Hyphessobrycon micropterus* clade, red circle *H. caru* sp. n., blue circles the other congeners within the clade, and black circle the clade *H. caru* sp. n. + *H. piorskii*.

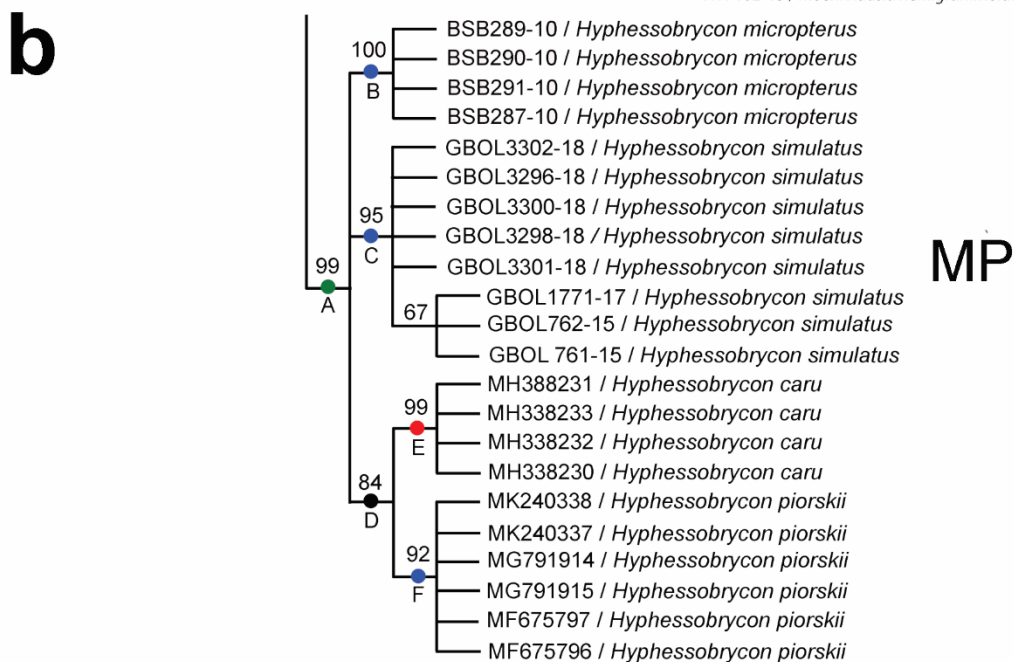
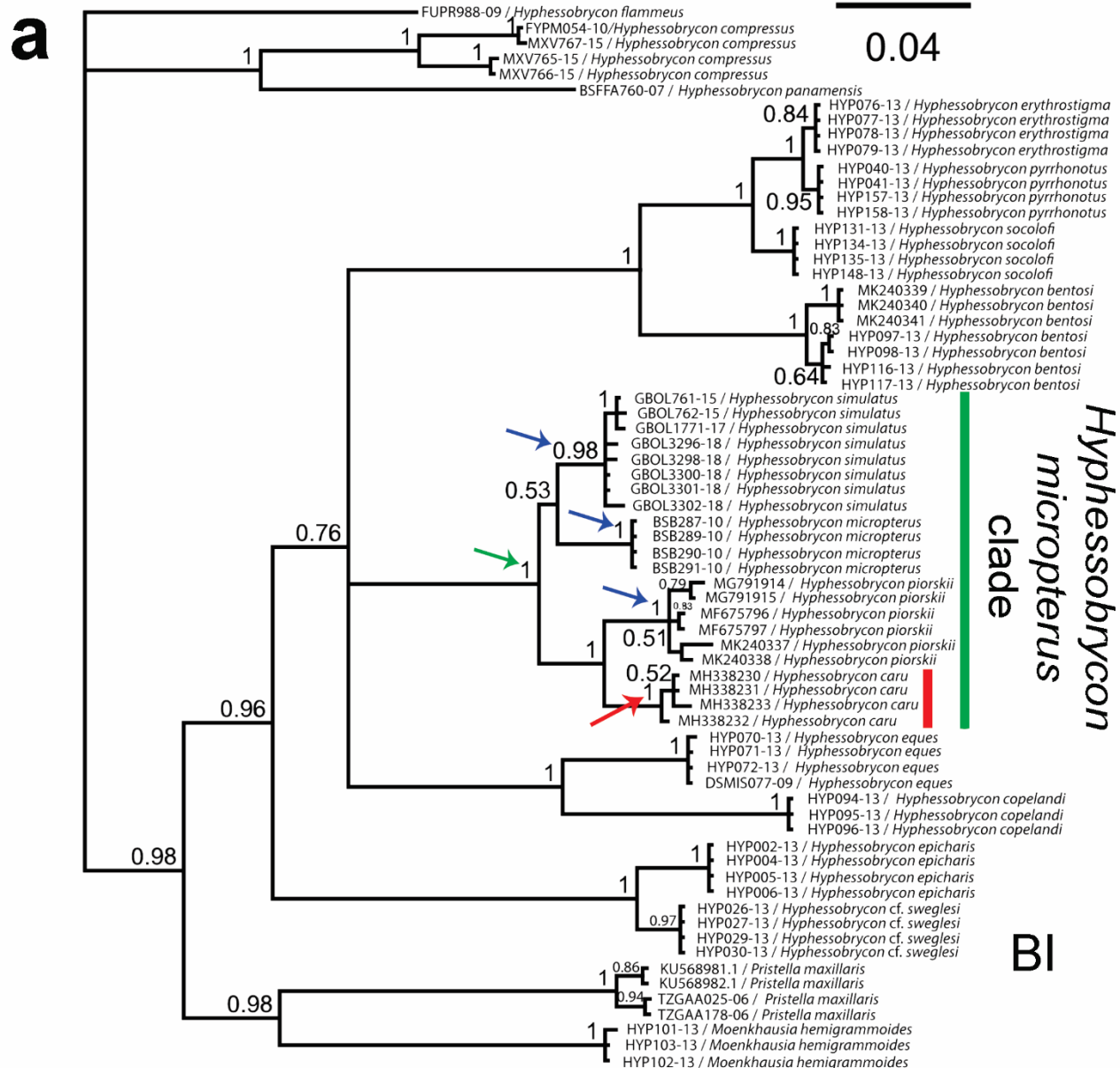
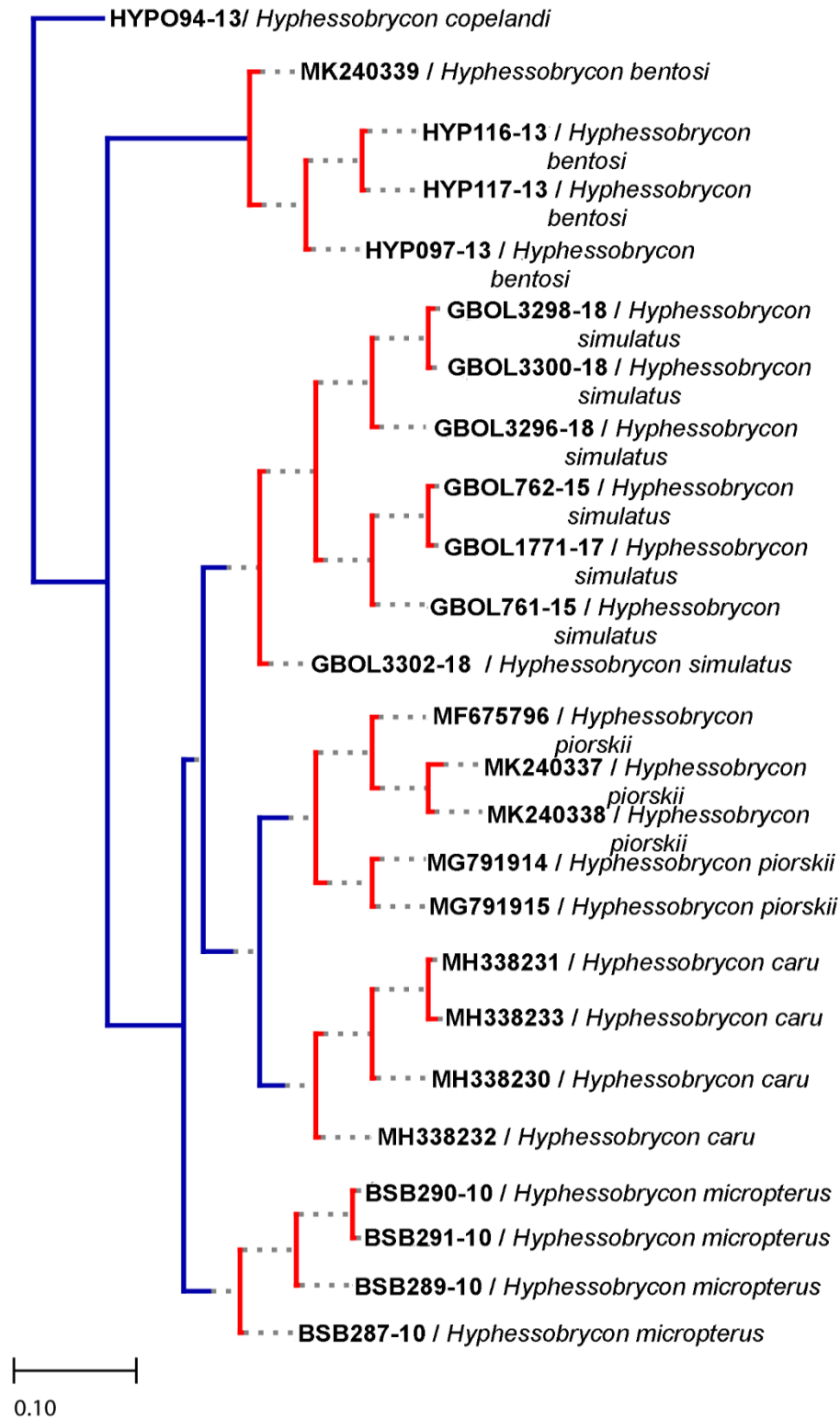


Figure 6. Species delimitation tree generated by the Bayesian Poisson Tree Processes (bPTP) model, using a fragment of the mitochondrial gene COI. The blue lines indicate branching processes among species, while red lines indicate branching processes within species.



Box 1. List of nucleotide substitutions (synapomorphies and autapomorphies) from each lineage (species) and some crucial points of the cladogram of the Fig. 5b.

A- COI 73 (C→T), COI 88 (T→C), COI 217 (C→T), COI 274 (C→T), COI 298(C→T), COI 334 (C→G), COI 338 (T→C), COI 370 (A→G), COI 418 (A→G), COI 433 (C→T), COI 439 (C→A), COI 457 (A→G), COI 469 (T→C), COI 478 (A→T), COI 559 (A→G), COI 562 (T→A), COI 592(A→G), COI 631 (A→T), COI 655 (A→C), COI 673 (A→C). **B -** COI 64 (A→C), COI 136 (A→G), COI 154 (C→T), COI 169 (C→T), COI 170 (C→T), COI 175 (T→C), COI 385 (C→T), COI 391 (C→A), COI 463 (T→G), COI 464 (G→A), COI 604 (T→C), COI 625 (A→G), COI 697 (A→G). **C-** COI 142 (A→G), COI 163 (T→C), COI 361 (T→C), COI 484 (T→C), COI 508 (T→C), COI 529 (A→G), COI 634 (T→C). **D-** COI 181 (A→C), COI 208 (A→G), COI 245 (C→T), COI 325 (T→C), COI 349 (T→C), COI 436 (A→T), COI 472 (A→G), COI 538 (C→T), COI 556 (T→C). **E-** COI 148 (C→T), COI 154 (C→T), COI 175 (T→C), COI 364 (G→A), COI 487 (T→C), COI 517 (A→G). **F-** COI 98 (C→T), COI 112 (T→C), COI 292 (A→G), COI 466 (C→T), COI 526 (C→A), COI 553 (T→C), COI 646 (A→G).

CAPÍTULO III

Integrative taxonomy reveals two new cryptic species of *Hyphessobrycon* Durbin, 1908 (Teleostei: Characidae) from the Maracaçumé and middle Tocantins River basins, Eastern Amazon region

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Integrative taxonomy reveals two new cryptic species of *Hyphessobrycon* Durbin, 1908 (Teleostei: Characidae) from the Maracaçumé and middle Tocantins River basins, Eastern Amazon region

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Abstract. Two new species, *H. frickei* sp. n., *H. geryi* sp. n., are herein described, based on seven different and independent species delimitation methods, and on molecular and morphological characters, making the hypothesis of these new species supported from an integrative taxonomy perspective. They belong to the “Rosy tetra” clade, which is characterized mainly by the presence of a dark brown or black blotch on dorsal-fin and absence of a midlateral stripe on the body. These two new species are distinguished from the other members of this clade mainly by the arrangement, shape and color pattern of humeral and dorsal-fin spots, as well as, by other characters related to scales counts and body pigmentation. The placement of the new species within the “Rosy tetra” clade were based on the combination of morphological character states mentioned above and corroborated by a molecular phylogenetic analysis using the mitochondrial gene cytochrome oxidase subunit 1. In addition, a new clade (here termed *Hyphessobrycon copelandi* clade) within the “Rosy tetra” clade is proposed based on molecular data, comprising *H. copelandi*., *H. frickei* sp. n., *H. geryi* sp. n. and a still undescribed species.

Our results corroborate the occurrence of cryptic speciation within the “Rosy tetra” clade, as suggested by previous studies.

Key words. ABGD, bPTP, DNA barcoding, GMYC “rosy tetra” clade

Introduction

Hyphessobrycon Durbin, 1908 is one of the most species-rich and widespread characid genus, comprising 160 valid species, occurring from southern Mexico to the Río de La Plata in Argentina (Fricke *et al.* 2020a). Despite its non-monophyletic status (Mirande 2010; Oliveira *et al.* 2011; Betancur-R *et al.* 2018; Guimarães *et al.* 2018; Mirande 2018; Guimarães *et al.* 2019), *Hyphessobrycon* is one of the genera within Characidae which species diversity has increased significantly in the past decades. Considering only the last decade, the number of species has risen from 109 to 160, an increase of about 30% in the number of valid species (Fricke *et al.* 2020a), and currently comprising approximately 15% of all characid species (Fricke *et al.* 2020b).

In the lack of a stable phylogenetic definition, many species have been described and were assigned to *Hyphessobrycon* based on its traditional diagnosis by Eigenmann (1918). A similar practice is commonly seen in other Characidae genera, such as, *Hemigrammus* Gill 1858 and *Knodus* Eigenmann 1911, for example (Javonillo *et al.* 2010; Oliveira *et al.* 2011). Although the number of *Hyphessobrycon* species has increased substantially, large knowledge gaps persists for the majority of species assigned to the genus, mainly regarding its phylogenetic position, such as: *Hyphessobrycon brumado* Zanata & Camelier 2010, *Hyphessobrycon frankei* Zarske & Géry 1997, *Hyphessobrycon negodagua* Lima & Gerhard 2001, and *Hyphessobrycon weneri* Géry & Uj 1987.

In an attempt to deal with the high diversity within the genus, species groups were proposed (Géry 1977; Weitzman & Palmer 1997; García-Alzate *et al.* 2008; Carvalho &

Malabarba 2015; Ota et al. 2020), such as: *Hyphessobrycon* groups a-f, “*callistus* group”, “Rosy tetra” clade, *Hyphessobrycon heterorhabdus*-group, *Hyphessobrycon sensu stricto*, and *Hyphessobrycon panamensis* species-group; some of them probably representing artificial operational assemblages to aid species identification, whereas others could represent potential monophyletic groups, with explicit putative synapomorphies (e.g. Castro-Paz et al. 2014; Bragança et al. 2015; Carvalho & Malabarba 2015; Guimarães et al. 2018, 2019).

Recent studies on Neotropical Characiformes, based on molecular data and species delimitation approaches, revealed a high cryptic diversity (e.g. Pereira et al. 2011; Melo et al. 2014; Benine et al. 2015; Benzaquem et al. 2015; Melo et al. 2016a, b, c; García-Melo et al. 2019). Similarly, three recently published papers (e.g. Castro-Paz et al. 2014; Guimarães et al. 2018, 2019) applied molecular species delimitation approaches to investigate the species diversity within the “Rosy tetra” clade, and they all agreed that its taxonomy should be better investigated, since it includes cryptic species.

In addition to the wide distribution and the presence of cryptic species, the taxonomic confuse history of that clade is one of the main challenges when studying its diversity and relationships. The type species of *Hemigrammus*, *H. unilineatus* (Gill 1858), and *Pristella* Eigenmann 1908, *P. maxillaris* (Ulrey 1894), have been historically considered as closely related or belonging to the “Rosy-tetra” clade, which also includes the type species of *Hyphessobrycon*, *H. compressus* (Meek 1904) (Weitzman & Palmer 1997; Carvalho & Malabarba 2015). Thus, *Hyphessobrycon* may be a putative synonym of *Hemigrammus* (Mirande 2010). However, further studies on the systematics of the species currently assigned to *Hyphessobrycon* and close related genera are still necessary to clarify this issue (Guimarães et al. 2018, 2019).

The aim of this study is to describe two new *Hyphessobrycon* cryptic species *sensu* Bickford *et al.* (2006), members of the “Rosy tetra” clade, from the Maracaçumé and middle Tocantins River basins, Eastern Amazon region, based on an integrative taxonomy approach. Furthermore, a new subclade, within the “Rosy tetra” clade, is herein proposed.

Materials and Methods

Taxa sampling, specimen collection, and preservation

Specimens were captured with manual trail-net (2 m long × 1.8m high; mesh size, 2mm) and euthanized in a buffered solution of ethyl-3-amino-benzoate-methanesulfonate (MS-222) at a concentration of 250 mg/l until completely ceasing opercular movements, according to animal welfare laws and guidelines (Leary *et al.* 2013; Close *et al.* 1996, 1997). Specimens selected for morphological analysis were fixed in formalin and left for 10 days, after which they were preserved in 70% ethanol. Molecular data were obtained from specimens that were euthanized, fixed and preserved in absolute ethanol. Specimens for morphological analysis are listed in type and comparative material lists. Specimens for molecular approaches are listed in (Table 1). Sequences from other species of *Hyphessobrycon* and allied species were obtained from the Barcode of Life Database (BOLD) and the National Center for Biotechnology Information (NCBI) databases (Table 1).

Morphological analysis

Measurements and counts were made according to Fink & Weitzman (1974). Vertical scale rows between the dorsal-fin origin and lateral line do not include the scale of the median predorsal series situated just anterior to the first dorsal-fin ray. Counts of

supraneurals, vertebrae, procurent caudal-fin rays, unbranched dorsal and anal-fin rays, branchiostegal rays, gill-rakers, premaxillary, maxillary, and dentary teeth were taken only from cleared and stained (C&S) paratypes, prepared according to Taylor & Van Dyke (1985). The four modified vertebrae that constitute the Weberian apparatus were not included in the vertebrae counts, and the fused PU1 + U1 was considered as a single element. Osteological nomenclature follows Weitzman (1962). Institutional abbreviations follow Fricke & Eschmeyer (2020).

DNA extraction, amplification and sequencing analyses

DNA was extracted from fin clips using Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's protocol. Fragments of the mitochondrial cytochrome c oxidase subunit 1 gene were amplified, using the universal primers designed by Ward *et al.* (2005) (FISHF1 5'-TCAACCAACCACAAAGACATTGGCAC-3' and FISHR1 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'). Polymerase chain reactions (PCR) comprised a total volume of 15 µl containing 1x Polymerase buffer, 1.5 mM MgCl₂, 200 µM dNTP, 0.2 uM of each primer, 1U of Taq Polymerase (Invitrogen), 100 ng of DNA template, and ultrapure water. The PCR cycles were as follows: 2 min at 94°C, followed by 35 cycles of 94°C for 30s, 54°C for 30s, and 72°C for 1 min, and 10 min at 72°C. Amplicons were purified using Illustra GFX PCR DNA and Gel Purification Kit (GE Healthcare Systems) and sequenced using the forward primer by an outsourced sequencing service at the University of São Paulo, using BigDye Terminator kit 3.1 Cycle Sequencing kit in ABI 3730 DNA Analyser (Applied Biosystems).

Data partition, evolution models, and alignment

The dataset cytochrome c oxidase subunit 1 (COI) (639 bp). Sequences were aligned using ClustalW (Chenna *et al.* 2003). The DNA sequences were translated into amino acids residues to test for the presence of premature stop codons or indels using the program MEGA 7 (Kumar *et al.* 2016). In the alignment, gaps were coded with a dash (–) and missing data with a question mark (?), but during analyses, both of them were treated as missing data. Measure Substitution Saturation tests were performed in DAMBE5 (Xia 2013) according to the algorithm proposed by Xia *et al.* (2003). The best-fit evolutionary model (GTR+G) was calculated, using the corrected Akaike Information Criterion (AICc) determined by the jModelTest 2.1.7 (Darriba *et al.* 2012).

Species concept, species delimitation, and diagnoses

The unified species concept is herein adopted by expressing the conceptual definition shared by all traditional species concepts – “species are (segments of) separately evolving metapopulation lineages” – disentangling operational criterion elements to delimit taxa from species concepts (de Queiroz 2005, 2007). According to this concept, species are treated as hypothetical units and could be tested (detected) by the application of distinct criteria (species delimitation methods) (de Queiroz 2005, 2007). It allows for any method to independently provide evidence about species limits and identities (de Queiroz 2005, 2007).

Seven distinct and independent methods relying on different operational criteria for species delimitation, based on morphological and molecular data, were implemented here: Population Aggregation Analysis (Davis & Nixon 1992); DNA barcoding, as proposed by Hebert *et al.* (2003a,b, 2004a, b); Automatic Barcode Gap Discovery

(Puillandre *et al.* 2012); a tree-based method as proposed by Wiens & Penkrot (2002) [following Sites & Marshall 2003]; a character-based DNA barcoding as proposed by Desalle *et al.* (2005); and two coalescent species delimitation methods termed the Bayesian implementation of the Poisson tree processes (Zhang *et al.* 2013), and General Mixed Yule Coalescent method (Fujisawa & Barraclough 2013).

All species delimitation methods here adopted, except PAA that relies only on morphological characters, were performed on cytochrome c oxidase subunit 1 (COI) sequences, since it is a mitochondrial gene with fast evolutionary rate, thus suitable and widely used for single locus species delimitation approaches (Avice 2000).

Population aggregation analysis (PAA)

The PAA (Davis & Nixon 1992) is a character-based method, in which species are delimited by a unique combination of morphological character states occurring in one or more populations (Costa *et al.* 2014). Basically, the occurrence of the same character state in individuals from different populations is evidence to the presence of gene flow between them, indicating that all correspond to the same species (lineage), whereas the presence of exclusive character states not shared between individuals belonging two distinct populations suggest that there is no gene flow between them, thus corresponding to distinct lineages.

Wiens and Penkrot analysis (WP)

WP is based on the direct inspection of haplotype trees generated from phylogenetic analysis having as terminals at least two individuals (haplotypes) of each focal species. In this method, the term 'exclusive' is used instead of monophyletic, since the term

monophyly is considered inapplicable below the species level (Wiens & Penkrot 2002). Clustered haplotypes with concordant geographic distribution forming mutual and well supported clades (exclusive lineages) are considered as strong evidence for species discrimination (absence of gene flow with other lineages). When haplotypes from the same locality fail to cluster together, there is potential evidence for gene flow with other populations (Wiens & Penkrot 2002). Haplotype tree statistical support was assessed by the posterior probability value, considered as significant at about 0.95 or higher (Alfaro & Holder 2006). When only one haplotype (specimen) from one putative population was available, the species delimitation was based on the exclusivity of the sister clade of this single haplotype, supported by significant values, allowing us to perform the test in populations with only one haplotype (Wiens & Penkrot 2002). In addition, the method allows recognition of nonexclusive lineages as species since their sister clades are exclusive and supported by significant values (Wiens & Penkrot 2002).

A Bayesian inference-based phylogenetic (BI) tree was estimated in MrBayes (Huelsenbeck & Ronquist 2001) plugin in Geneious 9.0.5 to reconstruct the evolutionary relationships among terminals using General Time Reversible (GTR+G) as evolutionary model. Bayesian tree inference was based in a chain length of 10 million, a burn-in length of 500,000 generations subsampling trees every 10,000 generations. We used a sequence of *Hyphessobrycon flammeus* Myers 1924 as outgroup.

Traditional DNA barcoding (DBC)

We used the Kimura-2-parameters model (K2P) (Kimura 1980) to estimate the pairwise genetic distances between species in MEGA 7 software (Kumar *et al.* 2016). We used DnaSP v6. (Rozas *et al.* 2003) to estimate the number of variable sites and haplotypes. We considered a cutoff of 2% as sufficient to discriminate species, since this threshold is

commonly inferred by species delimitations among Neotropical fish species based on COI (Jacobina *et al.* 2018).

Character-based DNA barcoding (CBB)

The CBB is similar to the population aggregation analysis proposed by Davis & Nixon (1992), but directed to nucleotides as an alternative method for diagnosing taxa through DNA barcodes, since the original method is based on subjective cut-off distance thresholds for the inference of species limits (Hebert *et al.* 2003a, b, 2004a, b). This method delimits species by the presence of a unique combination of nucleotides within a site shared by individuals of the same population or group of populations. In addition, species were diagnosed by nucleotide substitutions (Costa *et al.* 2014; Ottoni *et al.* 2019). Nucleotide substitutions among lineages were optimized in the Bayesian inference topology using PAUP 4 (Swofford 2002). Each nucleotide substitution is represented by its relative numeric position determined through sequence alignment with the complete mitochondrial genome of *Astyanax paranae* Eigenmann 1914 (KX609386.1:5503-7062), followed by the specific nucleotide substitution in parentheses. The results of this analysis are presented in Box 1 and molecular diagnosis sections.

General Mixed Yule Coalescent (GMYC)

The GMYC is a single locus coalescent phylogeny-based species delimitation approach that relies on the branches length to establish a threshold between speciation and coalescent processes (Fujisawa & Barraclough 2013). According to the method the dataset was reduced to include only unique haplotypes from *H. piorskii*, *H. caru*, *H. geryi* sp. n., *H. copelandi*, *H. frickei* sp. n. and *H. aff. copelandi*, and the outgroup was restricted to *Hyphessobrycon bentosi* Durbin, 1908. Sequences were aligned using ClustalW (Chenna *et al.* 2003). The best-fit evolutionary model (GTR+G) for the reduced dataset

was calculated using the corrected Akaike Information Criterion (AICc) determined by the jModelTest 2.1.7 (Darriba *et al.* 2012). The input ultrametric phylogenetic tree was performed in BEAST 1.8.4 (Drummond *et al.* 2012), with the following parameters: an uncorrelated relaxed clock with lognormal distribution, a Yule Process as tree prior with 10 million generations and sampling frequency of 1,000. The GMYC analysis was performed in the Exelixis Lab's server <https://species.h-its.org/gmyc/>.

Bayesian implementation of the poisson tree processes (bPTP)

The bPTP is another single locus coalescent phylogeny-based species delimitation method, but it distinguishes from other unilocus species delimitation coalescent approaches, such as GMYC, because it does not need an ultrametric tree (not relies on branch length to delimit species), avoiding errors and computer intensive processes (Zhang *et al.* 2013). The method assumes that more molecular variability (number of nucleotide substitutions) is expected between haplotypes from different species than within a species (Zhang *et al.* 2013), establishing a threshold between speciation and coalescent processes. The reduced dataset and the input phylogenetic tree for performing the bPTP was the same used in GMYC species delimitation method and previously detailed in the GMYC species delimitation method description. The bPTP analysis was performed in the Exelixis Lab's web server <http://species.h-its.org/ptp/>, following the default parameters except for a 20% burn-in.

Automatic Barcode Gap Discovery (ABGD)

The ABGD is a barcode species delimitation method that aims to establish a minimum barcode gap that probably corresponds to the threshold between interspecific and intraspecific processes (Puillandre *et al.* 2012). The major advantage of ABGD when

compared to the other barcode species delimitation barcode methods is that the Inference of the limit between interspecific and intraspecific processes (gap detection) are recursively applied to previously obtained groups to get finer partitions until there is no further partitioning, allowing a more refined search. Basically the ABGD analysis, indicates the number of groups (species) estimated relative to a large spectrum of p values (prior intraspecific values) in which a 0.1 value assumes a maximum intraspecific variability indicating that all sequences belongs to the only one species, and a 0.001 value that assumes a very small intraspecific variability indicating that each distinct haplotype represent a different species. After running the ABGD, additional molecular, morphological or ecological characters are needed to infer the correct number of species, following an integrative taxonomy perspective. The ABGD analysis was performed in the ABGD server website <https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html> following the default parameters.

RESULTS

Order Characiformes Regan, 1911

Family Characidae Latreille, 1825

Genus *Hyphessobrycon* Durbin, 1908

Hyphessobrycon frickei sp. nov. Guimarães, Brito, Bragança, Katz & Ottoni

(Figure 1; Table 1)

Holotype. CICCAA 02363, 18.8 mm SL, Brazil, Maranhão State, Maracaçumé municipality, Maracaçumé River, 2°3'14" S 45°57'16" W, 29 jan. 2017, Guimarães E. C., Brito P. S.

Paratypes. All from Brazil, Maranhão State, Maracaçumé municipality: CICCAA 02362, 10, 14.8-19.6 mm SL; CICCAA 02388, 5, C&S, 15.5-17.7 mm SL collected with holotype. UFRJ 6918, 1, 17.8 mm SL, stream tributary to the Maracaçumé River basin, 2°08'38" S 45°47'22" W, 29 jan. 2017, Guimarães E. C., Brito P. S.

Type Locality. *Hyphessobrycon frickei* sp. n. is only known from the Maracaçumé River basin, a coastal river in the Maranhão state, northeastern Brazil (Fig. 3).

Etymology. The new species is named *Hyphessobrycon frickei* after the ichthyologist Ronald Fricke in recognition to his enormous contribution to the ichthyology knowledge.

Morphological Diagnosis (PAA).

Hyphessobrycon frickei sp. nov. promptly differs from most of its congeners, except members of the “Rosy tetra” clade, by the presence of a dark brown or black blotch on dorsal-fin (vs. absence) and absence of a midlateral stripe on the body (vs. presence). The new species differs from most of its congeners within the “Rosy tetra” clade by possessing a conspicuous small slightly vertically elliptical humeral spot (Fig. 1) [vs inconspicuous vertically elongated humeral spot in *Hyphessobrycon bentosi* Durbin 1908, *H. caru* Guimarães, De Brito, Feitosa, Carvalho-Costa, Ottoni, 2018, *H. hasemani* Fowler 1913, *H. piorskii* Guimarães, De Brito, Feitosa, Carvalho-Costa, Ottoni, 2018; rounded humeral spot in *H. erythrostigma* (Fowler, 1943), *H. jackrobertsi* Zarske, 2014, *H. minor* Durbin, 1909, *H. pando* Hein, 2009, *H. paepkei* Zarske, 2014, *H. pyrrhonotus* Burgess, 1993, *H. roseus* (Géry, 1960), *H. socolofi* Weitzman, 1977, and *H. sweglesi* (Géry, 1961); horizontally or posteriorly elongated humeral spot in *H. epicharis* Weitzman & Palmer, 1997, *H. khardinae* Zarske, 2008, and *H. wernerii* Géry & Uj, 1987; vertical large

conspicuous humeral spot at least on males in *H. eques* (Steindachner, 1882), *H. haraldschultzi* Travassos, 1960, *H. micropterus* (Eigenmann, 1915), *H. megalopterus* (Eigenmann, 1915), *H. simulatus* (Géry, 1960) and *H. takasei* Géry, 1964; and absence of humeral spot in *H. compressus* (Meek, 1904), *H. dorsalis* Zarske, 2014, *H. georgettae* Géry, 1961, *H. pulchripinnis* Ahl, 1937, and *H. rosaceus* Durbin, 1909. (see. Guimarães *et al.* 2019: Fig. 2). The new species herein described differs from *H. copelandi* Durbin, 1908, and *H. haraldschultzi* Travassos, 1960 by the number of scales on lateral series [33-37 vs. 29-31, 28-30 lateral line scales, respectively]; and from *H. geryi* sp. nov. by the number of horizontal scale rows between lateral line and pelvic-fin origin [3 vs. 4-5, modally 4]. Furthermore, *H. frickei* sp. nov. differs from *H. geryi* sp. nov. by: the absence of conspicuous dark chromatophores on opercular zone (vs. presence); absence of conspicuous dark chromatophores on the region posterior to humeral spot (vs. presence, extending to the end of caudal peduncle); absence of a thin vertical line, formed by the concentration of dark chromatophores, at middle of humeral spot, extending one to two scales above and below the spot (vs. presence); and dorsal-fin base less pigmented (vs. conspicuous pigmentation on dorsal-fin base) (see Figs. 1 and 4).

Molecular diagnosis (CBB).

Hyphessobrycon frickei sp. nov. is member of the *H. copelandi* clade, possessing the following 25 nucleotide substitutions: COI 90 (C→A), COI 126 (A→G), COI 138 (C→T), COI 189 (C→T), COI 192 (T→A), COI 237 (C→T), COI 264 (T→C), COI 282 (C→T), COI 285 (C→A), COI 312 (T→C), COI 384 (C→T), COI 402 (A→G), COI 429 (A→G), COI 435 (A→G), COI 486 (T→C), COI 522 (A→G), COI 525 (C→T), COI 547 (C→T), COI 582 (T→C), COI 621 (T→C), COI 624 (A→G), COI 678 (T→C), COI 684 (A→C), COI 690 (T→C), COI 696 (A→G). In addition, it differs from *H.*

geryi sp. nov. by possessing the following nine nucleotide substitutions: COI 231 (T→C), COI 315 (A→G), COI 345 (A→C), COI 375 (G→A), COI 402 (G→A), COI 405 (T→C), COI 528 (A→G), COI 558 (A→G), COI 609 (A→G); and from *A. copelandi* by possessing the following eleven nucleotide substitutions: COI 126 (G→A), COI 141 (G→C), COI 291 (A→G), COI 300 (A→G), COI 345 (C→G), COI 366 (C→T), COI 435 (G→A), COI 510 (T→C), COI 633 (T→C), COI 657 (C→T), COI 672 (T→C).

Description

Morphometric data of holotype and paratypes are presented in Table 2. Body small (larger specimen with 19.6 mm of SL), laterally compressed, moderately deep, greatest body depth slightly anterior to dorsal-fin base; body profile straight and downward directed from the end of dorsal-fin to adipose-fin, straight or slightly convex between later point and origin of dorsal most procurent caudal-fin ray; dorsal profile of head convex from upper lip to vertical through eye; predorsal profile of body roughly straight, dorsal-fin base slightly convex, posteroventrally inclined; ventral profile of head convex from lower jaw to pelvic-fin origin; straight and posterodorsally slanted along anal-fin base; and slightly concave on caudal peduncle. Jaws equal, mouth terminal. Maxilla reaching vertical to anterior margin of pupil.

Premaxillary teeth in two rows. Outer row with two tricuspid teeth; inner row with 3(10), pentacuspid teeth and 3(7) or 2(3) tricuspid teeth. Maxilla with 2(5) or 3(6) tricuspid teeth. Dentary with five (10) larger pentacuspid teeth followed by five (8) or six (2) smaller tricuspid teeth tooth (Fig. 2).

Scales cycloid, three to eight radii strongly marked, circuli well-marked anteriorly, weakly-marked posteriorly; lateral line incompletely pored, with 7(6), 8(7)*, 9(3) or 10(1) perforated scales. Longitudinal scales series including lateral-line (perforated) scales 33(1), 34(1), 35(3), 36(7)* or 37(1). Longitudinal scales rows between dorsal-fin origin and lateral line 5(1) or 6(16)*. Horizontal scale rows between lateral line and pelvic-fin origin 3(17)*. Scales in median series between tip of supraoccipital spine and dorsal-fin origin 8(3) or 9(14). Circumpeduncular scales 10(12)*, 11(3) or 12(2).

Dorsal-fin origin at midbody. Base of last dorsal-fin ray at vertical through first third of anal-fin. Dorsal-fin rays ii + 9(10)*, iii + 9(5), ii+10(2). First dorsal-fin pterygiophore main body located behind neural spine of 4th vertebrae. Adipose-fin present. Anal-fin origin aligned with vertical line through middle of dorsal-fin, between 6th and 8th dorsal-fin rays. Anteriormost anal-fin pterygiophore inserting posterior to haemal spine of 11th vertebrae. Anal-fin origin aligned with vertical line through middle of dorsal-fin (between base of 6th and 8th dorsal-fin rays). Anal-fin iii+22(10)* or iii+23(7); Anterior anal-fin margin slightly convex, with anteriormost rays more elongate and slightly more thickened than remaining rays, forming a distinct lobe. Remaining rays smaller with straight distal margin. Pectoral-fin rays 12(17)*. Tip of pectoral-fin surpassing pelvic-fin base. Pelvic-fin rays 8(17)*, surpassing anal-fin origin. Caudal-fin forked, upper and lower lobes similar in size. Principal caudal-fin rays 11+10(10)* or 10+9(7); dorsal procurrent rays 8(2) or 9(3) and ventral procurrent rays 7(2) or 8(3).

Branchiostegal rays 4(5). First gill arch with 1(4), 2(1) hypobranchial, 11(1), 12(3), or 13(1) ceratobranchial, 1(5) cartilage between ceratobranchial and epibranchial, and 5(1)

or 6(4) epibranchial gill-rakers. Supraneurals 4(4) or 5(1). Precaudal vertebrae 11 (5) and caudal vertebrae 19 (5). Total vertebrae 30(5).

Colouration in alcohol (fig. 1)

Ground coloration light yellowish brown. Humeral spot conspicuous, slightly vertically elliptical. Flank with inconspicuous chromatophores, more concentrated on dorsal and ventral regions; middle region without or with inconspicuous chromatophores. Ventral region lacking dark brown chromatophores. Dark brown chromatophores present on dorsal portion of head and tip of snout, becoming sparser or absent on opercular region, and absent on cheek. Dorsal-fin ground coloration hyaline, with conspicuous black or dark brown spot located on anterior portion of fin, reaching about 6th ray, approximately between half to two thirds of fin depth. Dorsal-fin base with inconspicuous scattered chromatophores. Anal and caudal-fins hyaline. Anal and caudal fins with a darker, usually dark brown, posterior margin. Adipose-fin hyaline to light brown, with dark chromatophores at margin and posterior portion. Pectoral and pelvic-fins hyaline; pelvic-fin with variable amounts of dark brown pigmentation remaining depending on the specimen preservation state.

Sexual dimorphism

Bony hooks on fins is absent in all examined specimens. According to Malabarba & Weitzman (2003) the presence of bony hooks is a common dimorphic feature among characids species. Although this sexual dimorphism is not observed in all characid species, as in the case of the species described here.

. Colour pattern is also not sexually dimorphic.

Order Characiformes Regan, 1911

Family Characidae Latreille, 1825

Genus *Hyphessobrycon* Durbin 1908

***Hyphessobrycon geryi* sp. nov.** Guimarães, Brito, Bragança, Katz & Ottoni

Figures 4,5; Table 2

Holotype. CICCAA 02403, 27.8 mm SL, Brazil, Pará State, Marabá municipality, Flexeiras river, Tocantins River basin, 5°15'38"S 48°59'35"W, 20 set. 2016, Guimarães E. C., Costa. C. H.

Paratypes. All from Brazil: CICCAA 00722, 1, 25.6 mm SL collected with holotype. CICCAA 02358, 13, 15.9-19.5 mm SL, Pará State, Marabá municipality, Flexeiras river, Tocantins river basin, 5°15'38"S 48°59'35"W, 21 set. 2018, Guimarães E. C., Brito P. S; CICCAA 02359, 11, 16.9-19.2 mm SL, Pará State, Marabá municipality, Flexeiras river, Tocantins river basin, 5°15'38"S 48°59'35"W, 09 mar. 2018, Guimarães E. C., Brito P. S; CICCAA 02167, 11, 18.8-22.2 mm SL; Pará State, Bom Jesus do Tocantins, Jacundá river, Tocantins river basin, 5°15'1"S 48°48'58"W, 21 dez. 2017, Guimarães E. C., Brito P. S; CICCAA 2348, 1, 23.6 mm SL, Pará State, Bom Jesus do Tocantins, Jacundá river, Tocantins river basin, 5°15'1"S 48°48'58"W, 21 out. 2017, Guimarães E. C., Brito P. S; CICCAA 04800, 5 C&S, 21.1-23.1 mm SL, Pará State, Marabá municipality, Flexeiras river, Tocantins river basin, 5°15'38"S 48°59'35"W, 21 out. 2017, Guimarães E. C., Brito P. S; UFRJ 6919, 1, 17.9 mm SL, Pará State, Bom Jesus do Tocantins, Jacundá river, Tocantins river basin, 5°15'1"S 48°48'58"W, 21 set. 2018, Guimarães E. C., Brito P. S; CICCAA 01839, 4, 16.3-19.2 mm SL, Pará State, Marabá municipality, Tocantins River basin, 5°15'38"S 48°59'35"W, 10 jul. 2017, Guimarães E. C., Brito P; CICCAA 02402, 5

C&S, 18.0-18.9 mm SL, Pará State, Marabá municipality, Tocantins river basin, 5°15'38"S 48°59'35"W, 21 set. 2018, Guimarães E. C., Brito P. S; CICCAA 01958, 1, 26.1 mm SL, Maranhão State, igarapé papagaio, Tocantins River basin, 5° 8'59"S 48°22'33"W, 20 jan. 2014, Guimarães E. C.

Type Locality. Brazil, Pará State, Marabá municipality, Flexeiras river, Tocantins River basin, 5°15'38"S 48°59'35"W, 20 set. 2016, Guimarães E. C., Costa. C. H. (Fig. 3).

Etymology. The new species is named *Hyphessobrycon geryi* after the ichthyologist Jacques Géry in recognition to his enormous scientific contribution on Characiformes.

Morphological Diagnosis (PAA).

Hyphessobrycon geryi sp. nov. promptly differs from most of its congeners, except members of the “Rosy tetra” clade, by the presence of a dark brown or black blotch on dorsal-fin (vs. absence) and absence of a midlateral stripe on the body (vs. presence). The new species differs from most of its congeners from the “Rosy tetra” clade by possessing a conspicuous small slightly vertically elliptical humeral spot (Fig. 1) [vs inconspicuous vertically elongated humeral spot in *Hyphessobrycon bentosi* Durbin 1908, *H. caru* Guimarães, De Brito, Feitosa, Carvalho-Costa, Ottoni, 2018, *H. hasemani* Fowler 1913, *H. piorskii* Guimarães, De Brito, Feitosa, Carvalho-Costa, Ottoni, 2018; approximately rounded humeral spot in *H. erythrostigma* (Fowler, 1943), *H. jackrobertsi* Zarske, 2014, *H. minor* Durbin, 1909, *H. pando* Hein, 2009, *H. paepkei* Zarske, 2014, *H. pyrrhonotus* Burgess, 1993, *H. roseus* (Géry, 1960), *H. socolofi* Weitzman, 1977, and *H. sweglesi* (Géry, 1961); humeral spot horizontally or posteriorly elongated in *H. epicharis* Weitzman & Palmer, 1997, *H. khardinae* Zarske, 2008, and *H. weneri* Géry & Uj, 1987; vertical large conspicuous humeral spot at least on males in *H. eques* (Steindachner, 1882), *H. haraldschultzi* Travassos, 1960, *H. micropterus* (Eigenmann, 1915), *H. megalopterus* (Eigenmann, 1915), *H. simulatus* (Géry, 1960) and *H. takasei* Géry, 1964;

and absence of humeral spot in *H. compressus* (Meek, 1904), *H. dorsalis* Zarske, 2014, *H. georgettae* Géry, 1961, *H. pulchripinnis* Ahl, 1937, and *H. rosaceus* Durbin, 1909 (Guimarães *et al.* 2019: Fig. 2). The new species herein described differs from *H. copelandi* Durbin, 1908, *H. haraldschultzi* Travassos, 1960 by the number of lateral series of scales [33-37 vs. 29-31, 28-30 lateral line scales, respectively]; from *H. frickei* sp. nov. by the number of horizontal scale rows between lateral line and pelvic-fin origin [4-5, modally 4 vs. 3]; presence of conspicuous dark chromatophores on opercular zone (vs. absence); presence of conspicuous dark chromatophores on the region posterior to humeral spot, extending to the end of caudal peduncle (vs. absence); presence of a thin vertical line, formed by the concentration of dark chromatophores, at middle of humeral spot, extending one to two scales above and below the spot (vs. absence); and dorsal-fin base with conspicuous pigmentation (vs. less pigmented) (see Figs. 1 and 4).

Molecular diagnosis (CBB).

Hyphessobrycon geryi sp. nov. is member of the *H. copelandi* clade, possessing the following 25 nucleotide substitutions: COI 90 (C→A), COI 126 (A→G), COI 138 (C→T), COI 189 (C→T), COI 192 (T→A), COI 237 (C→T), COI 264 (T→C), COI 282 (C→T), COI 285 (C→A), COI 312 (T→C), COI 384 (C→T), COI 402 (A→G), COI 429 (A→G), COI 435 (A→G), COI 486 (T→C), COI 522 (A→G), COI 525 (C→T), COI 547 (C→T), COI 582 (T→C), COI 621 (T→C), COI 624 (A→G), COI 678 (T→C), COI 684 (A→C), COI 690 (T→C), COI 696 (A→G). In addition, it differs from the other species of this group by the following eight nucleotide substitutions: COI 225 (T→C), COI 228 (A→C), COI 321 (G→A), COI 522 (G→A), COI 534 (G→A), COI 543 (A→G), COI 589 (C→T), COI 615 (G→A).

Description

Morphometric data of holotype and paratypes are presented in Table 2. Body small (with maximum SL of 23.6 mm), compressed, moderately deep, greatest body depth slightly anterior to dorsal-fin base; body profile straight and downward directed from the end of dorsal-fin to adipose-fin, straight or slightly convex between later point and origin of dorsal most procurent caudal-fin ray; dorsal profile of head convex from upper lip to vertical through eye; predorsal profile of body roughly straight, dorsal-fin base slightly convex, posteroventrally inclined; ventral profile of head convex from lower jaw to pelvic-fin origin; straight and posterodorsally slanted along anal-fin base; and slightly concave on caudal peduncle; Jaws equal, mouth terminal. Maxilla reaching vertical to anterior margin of pupil.

Premaxillary teeth in two rows. Outer row with two tricuspid teeth; inner row with 5(2), 6(3) tricuspid teeth. Maxilla with 2(2), 3(2) or 4(1) pentacuspoid teeth. Dentary with five (3) or six (2) larger tricuspid teeth followed by five (4) or six (1) smaller pentacuspoid tooth (Fig. 6).

Scales cycloid, three to eight radii strongly marked, circuli well-marked anteriorly, weakly-marked posteriorly; lateral line incompletely pored, with 7(31)* or 8(21) perforated scales. Longitudinal scales series including lateral-line scales 34(4), 35(2), 36(43)* or 37(1). Longitudinal scales rows between dorsal-fin origin and lateral line 5(46) or 6(4). Horizontal scale rows between lateral line and pelvic-fin origin 4(50) or 5(2). Scales in median series between tip of supraoccipital spine and dorsal-fin origin 9(35) or 10(15). Circumpeduncular scales 12(52).

Dorsal-fin origin at midbody. Base of last dorsal-fin ray at vertical through first third of anal-fin. Dorsal-fin rays $ii + 9(50)^*$ or $iii + 9(2)$. First dorsal-fin pterygiophore main body located behind neural spine of 4th vertebrae. Adipose-fin present. Anal-fin origin aligned with vertical line through middle of dorsal-fin, between 6th and 8th dorsal-fin rays. Anteriormost anal-fin pterygiophore inserting posterior to haemal spine of 11th vertebrae. Anal-fin origin aligned with vertical line through middle of dorsal-fin (between base of 6th and 8th dorsal-fin rays). Anal-fin $iii+22(40)^*$ or $iii+23(12)$; Anterior anal-fin margin slightly convex, with anteriormost rays more elongate and slightly more thickened than remaining rays, forming a distinct lobe. Remaining rays smaller with straight distal margin. Pectoral-fin rays 12(52) total rays. Tip of pectoral-fin surpassing pelvic-fin base. Pelvic-fin rays 8(52) total rays, surpassing anal-fin origin. Caudal-fin forked, upper and lower lobes similar in size. Principal caudal-fin rays $11+10(30)$ or $10+9(22)$; dorsal procurrent rays 8(8) or 9(2) and ventral procurrent rays 7(3) or 8(7).

Branchiostegal rays 10(10). First gill arch with 1(8), 2(2) hypobranchial, 11(2), 12(8) ceratobranchial, 1(10) cartilage between ceratobranchial and epibranchial, and 5(2) or 6(8) epibranchial gill-rakers. Supraneurals 4(8) or 5(2). Precaudal vertebrae 11 (10) and caudal vertebrae 19 (10). Total vertebrae 30(10).

Colouration in alcohol (fig. 4)

Ground coloration light yellowish brown. Humeral spot conspicuous, slightly vertically elliptical, with a thin vertical line, formed by the concentration of dark chromatophores, at middle of humeral spot, extending one to two scales above and below the humeral spot. Flank with conspicuous chromatophores scattered mainly on middle region, from just after humeral spot, reaching caudal peduncle end. Ventral region lacking dark brown chromatophores. Dark brown chromatophores present on head, more concentrated on

dorsal portion, tip of snout, and opercular region, becoming sparser on cheek. Dorsal-fin ground coloration hyaline, with conspicuous black or dark brown spot located on anterior portion of fin, reaching about 6th ray, approximately between half to two thirds of fin depth. Conspicuous pigmentation on dorsal-fin base formed by concentration of conspicuous chromatophores. Anal and caudal fins hyaline. Anal and Caudal-fin with a darker, usually dark brown, posterior margin. Adipose-fin hyaline to light brown, with dark chromatophores at margin and posterior portion. Pectoral and pelvic-fins hyaline; pelvic-fin with variable amounts of dark brown pigmentation remaining depending on the specimen preservation state.

Color in life (fig. 5)

Color pattern similar to coloration of preserved specimens, with a more translucent body. Ground coloration translucent, white to grey, with orange pigmentation on vertebrae region, and usually with orange chromatophores. Ventral region anterior to anal-fin origin lighter.

Chromatophores on humeral region black. Head darker than flank, especially on opercular and dorsal regions; ventrally lighter. Conspicuous black spot on dorsal-fin, with yellow or white pigmentation on dorsal and ventral margins of spot; rest of dorsal fin hyaline. Anal-fin base with light red chromatophores, with different degrees of intensity, with milk white pigmentation on anterior tip of anal-fin, reaching between first to second rays. Adipose-fin hyaline, with light red chromatophores mainly at base, and black chromatophores at margin. Pectoral and pelvic fins hyaline, with some sparser dark brown chromatophores, more concentrated at pelvic-fin, and with milk white pigmentation on anterior tip of pelvic-fin. Caudal fin with red pigmentation on almost fin, with an inconspicuous light brown margin.

Sexual dimorphism

Bony hooks on fins is absent in all examined specimens. According to Malabarba & Weitzman (2003) the presence of bony hooks is a common dimorphic feature among characids species. Although this sexual dimorphism is not observed in all characid species, as in the case of the species described here. Colour pattern is also not sexually dimorphic.

Species Delimitation

WP and CBB

The BI phylogenetic analysis supported a clade comprising *Hyphessobrycon geryi* sp. n., *H. copelandi*, *H. frickei* sp. n., and *H. aff. copelandi*, hereafter termed *Hyphessobrycon copelandi* clade, with maximum posterior probability value (posterior probability = 1). *Hyphessobrycon frickei* sp. n and *H. geryi* sp. n. haplotypes formed two exclusive clades with maximum posterior probability value (posterior probability = 1), as well as, *H. copelandi* and *H. aff. copelandi*. The nucleotide substitutions supporting these lineages within the *H. copelandi* clade are presented in Figure 7 and box 1 of supplementary material. The combination of nucleotide substitutions diagnosing *H. frickei* sp. n. and *geryi* sp. n. are presented in the molecular diagnosis section.

DBC

The alignment comprised a total of 26 haplotypes. After trimming, the final alignment yielded 639 base pairs with 159 polymorphic sites. Average genetic distances were 17%, with the highest values between *H. epicharis* and *H. erythrostigma* (23.4%), while the lowest value of (0.08%) was between *H. pyrrhonotus* and *H. erythrostigma* (Table 3). *Hyphessobrycon frickei* sp. n. and *H. geryi* sp. n. are divergent on average 17%, from the

other taxa, with a minimum distance of 3.5% and 4.4%, respectively, to *H. copelandi*, and 4.7% of divergence between them (Table 3), thus both species being recovered by this genetic distance method.

GMYC and bPTP

Both unilocus coalescent species delimitation methods delimited the same eight lineages (species): *H. caru*, *H. piorskii*, *H. geryi* sp. n., *H. copelandi*, *H. frickei* sp. n. and *H. aff. copelandi*; in addition to the species included as outgroup, *H. bentosi*, that was splitted in two distinct lineages (Fig. 8).

ABDG

In the ABDG species delimitation method, the same ingroup taxa as delimited in the coalescent species delimitation methods have been defined comprising: *H. caru*, *H. piorskii*, *H. geryi* sp. n., *H. copelandi*, *H. frickei* sp. n. and *H. aff. copelandi*. In contrast to the coalescent methods results, all haplotypes from *H. bentosi*, the outgroup taxa, were recognized as a single species. The same seven groups (species) were delimited between P values ranging from 0.0017 and 0.0215 (Fig. 8).

Discussion

Recent DNA-based studies suggest that tropical faunas contain a large proportion of undescribed species (e.g. Roca *et al.* 2001; Hrbek *et al.* 2014; Poulakakis *et al.* 2015; Murphy *et al.* 2016; Bittencourt *et al.* 2019; Guimarães *et al.* 2018, 2019; de Brito *et al.* 2018, 2019). This fact is more evident in freshwater fishes, since freshwater systems are isolated, hampering the migration of species from one river system to another. Thus, favoring speciation, in many cases cryptic speciation, which are common among Neotropical fish lineages, as evidenced and revealed by works dealing with species delimitation molecular approaches (e.g. Costa & Amorim 2011; Costa *et al.* 2012, 2014, 2017; Pereira *et al.* 2011; Benzaquem *et al.* 2015; Melo *et al.* 2014, 2016a, b, c; Amorim

2018; Mattos & Costa 2018; Carvalho *et al.* 2018; Guimarães *et al.* 2018, 2019; Rosso *et al.* 2018; Ottoni *et al.* 2019; de Santana *et al.* 2019). Recently, the presence of cryptic species and a greater biodiversity have been also evidenced for the “Rosy tetra” clade (e.g. Castro-Paz *et al.* 2014; Guimarães *et al.* 2018, 2019), and is herein corroborated, with the description of two new species belonging to this clade, based on seven different species delimitations methods.

In the present research we suggest a new clade within the “Rosy tetra”, the *Hyphessobrycon copelandi* clade, comprising four species: *Hyphessobrycon copelandi*, with its type locality at Tabatinga, Amazonas, Brazil, close to the border with Peru and Bolivia (Eigenmann 1908); the two new species herein described, *Hyphessobrycon frickei* and *H. geryi* from the Maracaçumé River basin in northeastern Brazil and the middle Tocantins River basin in east Amazon, Brazil, respectively; and an undescribed species from the lower Rio Negro basin (here called *H. aff. copelandi*). The *Hyphessobrycon copelandi* clade was corroborated by maximum node support (see Fig. 7), as well as, by 25 synapomorphic nucleotide substitutions (see Fig. 7 and box 1).

Hyphessobrycon frickei and *H. geryi* were identified as new species by seven different and independent methods of species delimitation (PAA, DBC, WP, CBB, bPTP, GMYC and ABGD), relying on different sources of characters (morphology and DNA), different criteria and assumptions; under an Integrative Taxonomy perspective (Sytsma & Schaal 1985; Dayrat 2005; Goldstein & Desalle 2010; Padial *et al.* 2010). Both species are distinguished from all its congeners mainly by the arrangement, shape and color pattern of humeral and dorsal-fin spots, as well as, by other characters related to scales counts and body pigmentation (see PAA – Diagnosis). In our bayesian inference phylogenetic

analysis (Figure 7), haplotypes belonging to both species formed two exclusive clades with maximum posterior probability value (posterior probability = 1) (WP), as well as, the other two species within the *Hyphessobrycon copelandi* clade. Furthermore, the minimum COI genetic distance (DBC) of *Hyphessobrycon frickei* and *H. geryi* when compared to *H. copelandi* were 3.5% and 4.4%, respectively, and 4.7% of divergence between them (see table 4). Considering this value, *Hyphessobrycon frickei* and *H. geryi* genetic divergence is greater than that commonly inferred by species delimitations among Neotropical fish species (2%) based on COI (Jacobina *et al.* 2018). Moreover, *Hyphessobrycon frickei* and *H. geryi* were also molecularly diagnosed by synapomorphic nucleotide substitutions, as well as by an accessory combination of other nucleotide substitutions (see CBB - molecular diagnosis sections, fig. 7 and box1). At last, both coalescent species delimitation methods (GMYC and bPTP) corroborated these two new species, as well as, the Automatic Barcode Gap Discovery (ABGD). The same result found by all the methods applied here only reinforces the hypothesis that these species are in fact new to science.

Similar results from previous studies (e.g Castro-Paz *et al.* 2014; Guimarães *et al.* 2018, 2019) agrees with our hypothesis of the existence of cryptic species within the “Rosy tetra” clade, and also suggests that its species diversity is still underestimated. We would like to highlight the paper published by Guimarães *et al.* (2018, 2019) that also proposed and evidenced a new subclade within the “Rosy tetra” clade, the *Hyphessobrycon micropterus* clade, revealing the existence of cryptic species and a greater diversity than previously known within the group.

Conclusion

The present paper highlights the importance of species delimitation through an integrative framework allowing to solve taxonomic and classification problems on *Hyphessobrycon* taxonomy, and estimating the diversity of this group with accuracy, being crucial for futures studies on phylogeny, phylogeography, ecology, conservation and biogeography. We consider that difficulties to recognize the boundaries at the species or genus levels can be overcome through the adoption of integrative studies and perspectives, dealing with multiple criteria and character sources to make taxonomic decisions.

Comparative material

***H. bentosi*:** MCZ 20842, 15 spcms (Syntypes); USNM 120270, 3 spcms (Syntypes); CAS 42682, 3 spcms (Syntypes), Óbidos; CICCAA04788, 100, spcms, Óbidos municipality, Amazon river basin, Pará state, Brazil; CICCAA 04789, 30 spcms (C&S) spcms, Óbidos municipality, Amazon river basin, Pará state, Brazil. CICCAA 04790, 131, spcms, Óbidos municipality, Amazon river basin, Pará state, Brazil. ***H. caru*:** CICCAA 02286, 1 spcm (Holotype); CICCAA 00706, 37 spcms (Paratypes); CICCAA 0709, 12 spcms (C&S) (Paratypes); LIOP.UFAM 1009, 1 spcm (Paratypes), CICCAA00707, 3 spcms (Paratypes); CICCAA 00708, 2 spcms (Paratypes); UFRJ11745, 1 spcm (Paratypes), Buriticupu municipality, Buritizinho river, Mearim river basin, Maranhão state, Brazil. ***H. compressus*:** BMNH 1905.12.6.4-5, 2 spcms (Paratypes), Oaxaca state. México. ***H. copelandi*:** CAS 42683, 5 spcms (Syntypes); MCZ 20771, 88 spcms (Syntypes); USNM 120271, 6 spms (Syntypes), Tabatinga. CICCAA04834, 18 spcms, Óbidos municipality, Amazon river basin, Pará state, Brazil. ***H. eques*:** MMW 95056, 1 spcm (Lectotype); NMW 62693, 6 spcms (Paralectotypes); NMW59810, 2 spcms (Paralectotypes), Villa Bela, Óbidos; CICCAA 00715, 4 spcms (C&S); CICCAA 00710, 51 spcms, Tombos municipality, Paraíba do Sul river basin, Minas Gerais state, Brazil; CICCAA00300, 24

spcms; CICCAA 00699, 7 spcms, Itaberaba municipality, São Francisco river basin, Bahia state, Brazil; CICCAA03447, 5 spcms; CICCAA03448, 6 spcms, Corumbá municipality, Paraguay river basin, Mato Grosso do Sul state, Brazil. CICCAA 04791, 240; CICCAA04792, 30 spcms (C&S); CICCAA04793, 6 spcms, Santarem municipality, Tapajós river basin, Pará State, Brazil. *H. erythro stigma*: ANSP 70208, 1 spcm (Holotype), Peru and Brazil. *H. epicharis*: FMNH100609, 1 spcm (Paratypes), Baria river, Amazonas, Venezuela. *H. haraldschultzi*: CICCAA 00873, 20 spcms, Ilha do Bananal municipality, Javaés river, Tocantins state, Brazil. *H. hasemani*: ANSP 39230, 1 spcm (Holotype), Guajaramirim municipality, Madeira river, Rondônia state, Brazil. *H. jackrobertsi*: MTD F 33042, 1 spcm (Holotype), Peru, vermutlich Umgebung von Iquitos; MTD F 33043-33051, 9 spcms, Peru, vermutlich Umgebung von Iquitos; MTD F 33149-33158, 10 spcms, Peru, Loreto, río Pastaza, río Chuinda, Lago de Rimachi. *H. micropterus*: FMNHH 57916, 1 spcm (Holotype), Lagoa de Porto, Minas Gerais state, Brazil. FMNH 57917, 14 spcms (Paratypes) Lagoa de Porto; FMNH 57918, 9 spcms (Paratypes); Santa Rita, São Francisco river basin; FMNH 57920, 25 spcms (Paratypes), Pirapora; FMNH 57920, 1 spcm (Paratypes), Rio Salitre; FMNH 57921, 4 spcm (Paratypes), Boqueirao, near mouth of Rio Preto. *H. paepkei*: MTD F 33023, 1 spcm (Holotype), Brasilien, Umgebung von Manaus. *H. piorskii*: CICCAA 00695, 1 spcm (Holotype); CICCAA 00430, 15 spcms (Paratypes); CICCAA 00431, 21 spcms (Paratypes); CICCAA 00696, 15 spcms (Paratypes); CICCAA 00697, 16 spcms (C&S) (Paratypes); CICCAA 00698, 6 spcms, 1 spcm (C&S) (Paratypes); CICCAA 00750, 9 spcms (Paratypes); CICCAA01654, 1 spcm (Paratypes); CICCAA 03385, [donated from CPUFMA 171664], 15 spcms (Paratypes); UFRJ 11553, 6 spcms (Paratypes), stream at the Anapurus municipality, Munim river, Maranhão state, Brazil. CICCAA 00089, 1 spcm (C&S) (Paratypes); CICCAA 00881, 1 spcm (Paratype); CICCAA 01563, 1 spcm

(Paratype); stream at Mata de Itamacaoca, Chapadinha municipality, Munim river, Maranhão state, Brazil. CICCAA 01382, 5 spcms (Paratypes); CICCAA 02008, 12 (C&S) spcms (Paratype), stream at Mata Fome, Barreirinhas municipality, Preguiças river, Maranhão state, Brazil. *H. pyrrhonotus*: MZUSP 45714, 1 spcm (Holotype), Eréré river, Brazil. *H. rosaceus*: FMNH 52791, 1 spcm (Holotype), Gluck Island, Essequibo River, Guyana. *H. werneri*: MZUSP 42365, 1 spcm (Holotype), Santa Maria do Pará and São Miguel do Guamá municipality, Guamá river, Pará state, Brazil. CICCAA 00751, 1 spcm, Paragominas municipality, Candiru river, Pará state, Brazil. *H. socolofi*: MZUSP 13181, 1 spcm (Holotype), Barcelos municipality, Negro river, Amazonas state, Brazil.

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Fig. 1. *Hyphessobrycon frickei* sp. n. CICCAA 02363, holotype, 02363, 18.8 mm SL;
Brazil: Maranhão State: Maracaçumé River basin.



Fig. 2. *Hyphessobrycon frickei* sp. n. CICCAA 02388, 17.7 mm SL; jaw suspensory: **a** -
Premaxillary. **b** - Maxilla. **c** - Dentary. Scale 1 mm

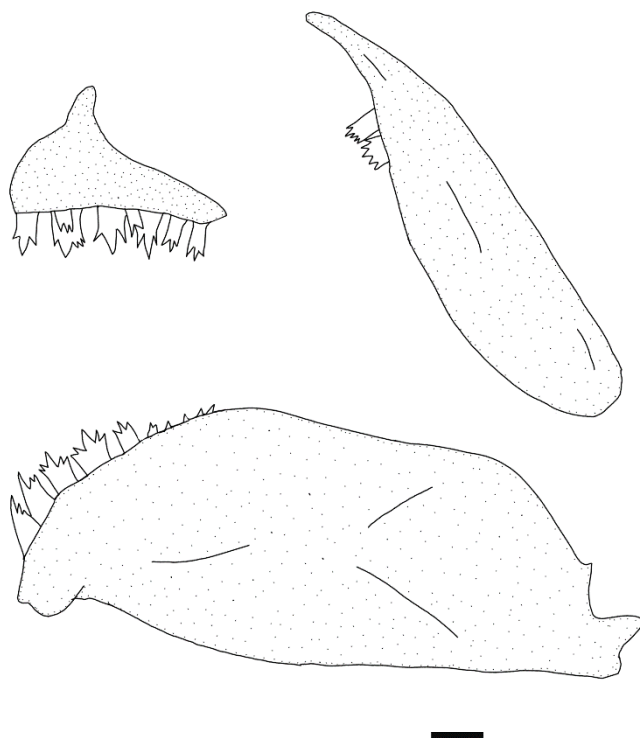


Fig. 3. Geographical distribution of *Hyphessobrycon frickei* sp. n. and *H. geryi* sp. nov.

Red star denotes Holotype and blue circles denote paratypes of *H. frickei* sp. n. and white star denotes Holotype and the black circles denote paratype of *H. geryi* sp. nov.

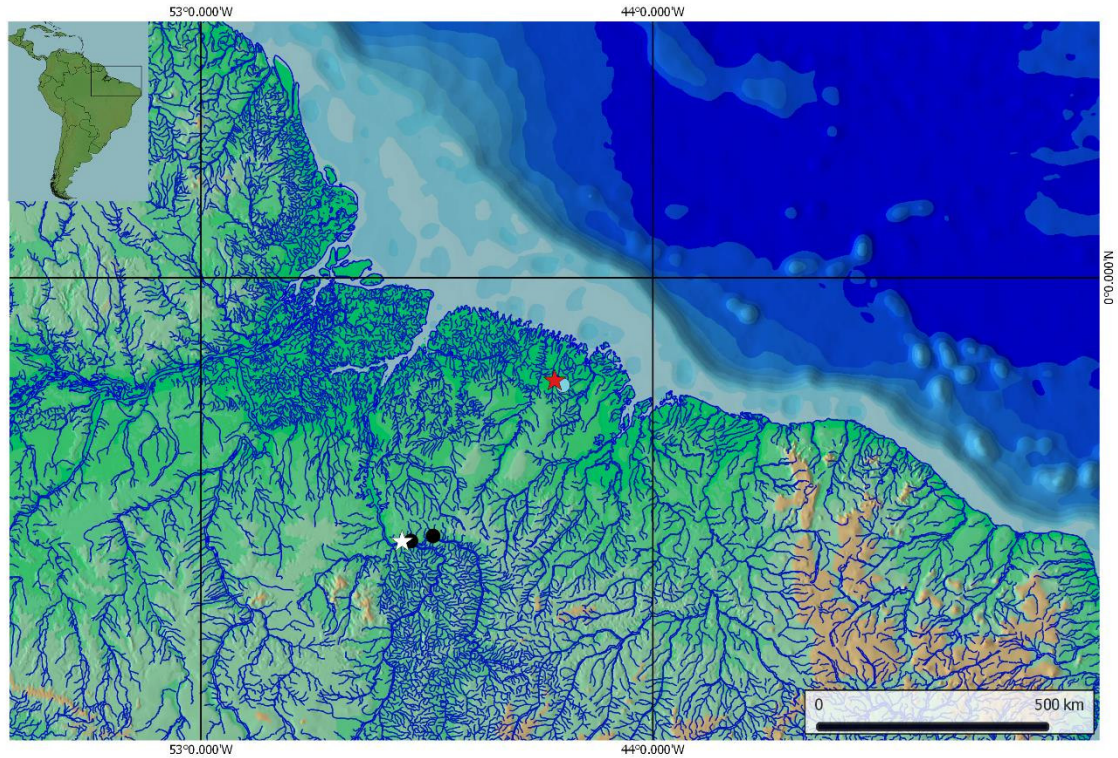


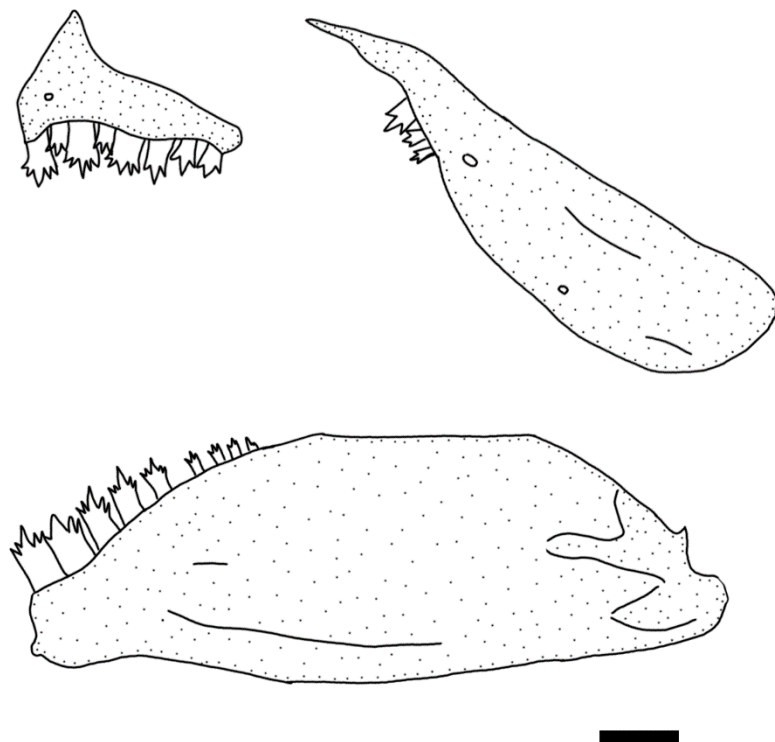
Fig. 4. *Hyphessobrycon geryi* sp. n. CICCAA 02403, holotype, 28.3 mm SL; Brazil: Maranhão State: Lower Tocantins River basin.



Fig. 5. *Hyphessobrycon geryi* sp. n. CICCAA 02348, paratype, 22.7 mm SL; Brazil: Maranhão State: Lower Tocantins River basin; living specimen photographed immediately after Collection.



Fig. 6. *Hyphessobrycon geryi* sp. n. CICCAA 02402, 23.2 mm SL; jaw suspensory: **a** - Premaxillary. **b** - Maxilla. **c** – Dentary. Scale 1 mm



A

FUPR988-09 | *Hyphessobrycon flammeus*
FYPM054-10 | *Hyphessobrycon compressus*
MXV767-15 | *Hyphessobrycon compressus*
MXV765-15 | *Hyphessobrycon compressus*
MXV766-15 | *Hyphessobrycon compressus*
BSFFA760-07 | *Hyphessobrycon panamensis*
HYP040-13 | *Hyphessobrycon pyrrhonotus*
HYP041-13 | *Hyphessobrycon pyrrhonotus*
HYP157-13 | *Hyphessobrycon pyrrhonotus*
HYP158-13 | *Hyphessobrycon pyrrhonotus*
HYP076-13 | *Hyphessobrycon erythrostigma*
HYP077-13 | *Hyphessobrycon erythrostigma*
HYP078-13 | *Hyphessobrycon erythrostigma*
HYP079-13 | *Hyphessobrycon erythrostigma*
HYP131-13 | *Hyphessobrycon socofli*
HYP134-13 | *Hyphessobrycon socofli*
HYP148-13 | *Hyphessobrycon socofli*
HYP135-13 | *Hyphessobrycon socofli*
MK409672 | *Hyphessobrycon bentosi*
MK240340 | *Hyphessobrycon bentosi*
MK240341 | *Hyphessobrycon bentosi*
MK240338 | *Hyphessobrycon bentosi*
HYP097-13 | *Hyphessobrycon bentosi*
HYP098-13 | *Hyphessobrycon bentosi*
HYP116-13 | *Hyphessobrycon bentosi*
HYP117-13 | *Hyphessobrycon bentosi*
MG791915 | *Hyphessobrycon pioskii*
MG791914 | *Hyphessobrycon pioskii*
MF765796 | *Hyphessobrycon pioskii*
LMF765797 | *Hyphessobrycon pioskii*
MK240337 | *Hyphessobrycon pioskii*
MK240338 | *Hyphessobrycon pioskii*
MH338230 | *Hyphessobrycon caru*
MH338231 | *Hyphessobrycon caru*
MH338232 | *Hyphessobrycon caru*
MH338233 | *Hyphessobrycon caru*
BSB287-10 | *Hyphessobrycon micropterus*
BSB288-10 | *Hyphessobrycon micropterus*
BSB289-10 | *Hyphessobrycon micropterus*
BSB290-10 | *Hyphessobrycon micropterus*
GBOL1771-17 | *Hyphessobrycon simulatus*
GBOL3298-18 | *Hyphessobrycon simulatus*
GBOL3300-18 | *Hyphessobrycon simulatus*
GBOL3301-18 | *Hyphessobrycon simulatus*
GBOL3302-18 | *Hyphessobrycon simulatus*
DSMS077-09 | *Hyphessobrycon eques*
HYP070-13 | *Hyphessobrycon eques*
HYP071-13 | *Hyphessobrycon eques*
HYP072-13 | *Hyphessobrycon eques*
MK409673 | *Hyphessobrycon geryi*
MK409674 | *Hyphessobrycon geryi*
MK409675 | *Hyphessobrycon geryi*
MK409676 | *Hyphessobrycon frikeli*
MK409677 | *Hyphessobrycon frikeli*
MK409678 | *Hyphessobrycon frikeli*
HYP09413 | *Hyphessobrycon copelandi*
HYP09513 | *Hyphessobrycon copelandi*
HYP09613 | *Hyphessobrycon copelandi*
HYP017-13 | *Hyphessobrycon aff. copelandi*
HYP018-13 | *Hyphessobrycon aff. copelandi*
HYP016-13 | *Hyphessobrycon aff. copelandi*
HYP019-13 | *Hyphessobrycon aff. copelandi*
HYP002-13 | *Hyphessobrycon epicharis*
HYP004-13 | *Hyphessobrycon epicharis*
HYP005-13 | *Hyphessobrycon epicharis*
HYP006-13 | *Hyphessobrycon epicharis*
HYP026-13 | *Hyphessobrycon cf. sweglesii*
HYP027-13 | *Hyphessobrycon cf. sweglesii*
HYP029-13 | *Hyphessobrycon cf. sweglesii*
HYP030-13 | *Hyphessobrycon cf. sweglesii*
GBOL759-15 | *Hyphessobrycon roseus*
GBOL1785-17 | *Hyphessobrycon roseus*
GBOL3336-18 | *Hyphessobrycon roseus*
GBOL760-15 | *Hyphessobrycon roseus*
KUS68979.1 | *Pristella maxillaris*
KUS68982.1 | *Pristella maxillaris*
TZGAA025-06 | *Pristella maxillaris*
TZGAA178-06 | *Pristella maxillaris*
HYP103-13 | *Moenkhausia hemigrammoides*
HYP102-13 | *Moenkhausia hemigrammoides*
HYP101-13 | *Moenkhausia hemigrammoides*

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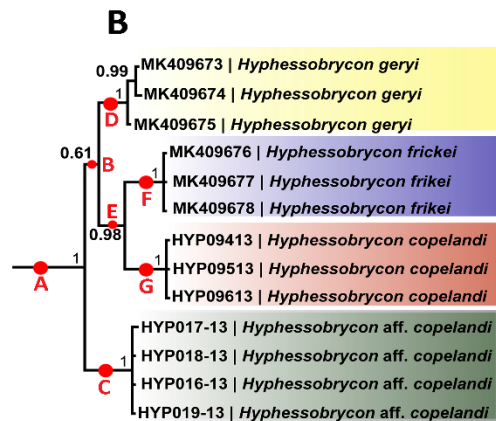


Fig. 8. Topology of the ultrametric tree performed in BEAST 1.8.4 including unique haplotypes summarizing the results of GMYC, bPTP and ABGD. Numbers above and below branches are posterior probability values. Star indicates the *Hyphessobrycon copelandi* clade.

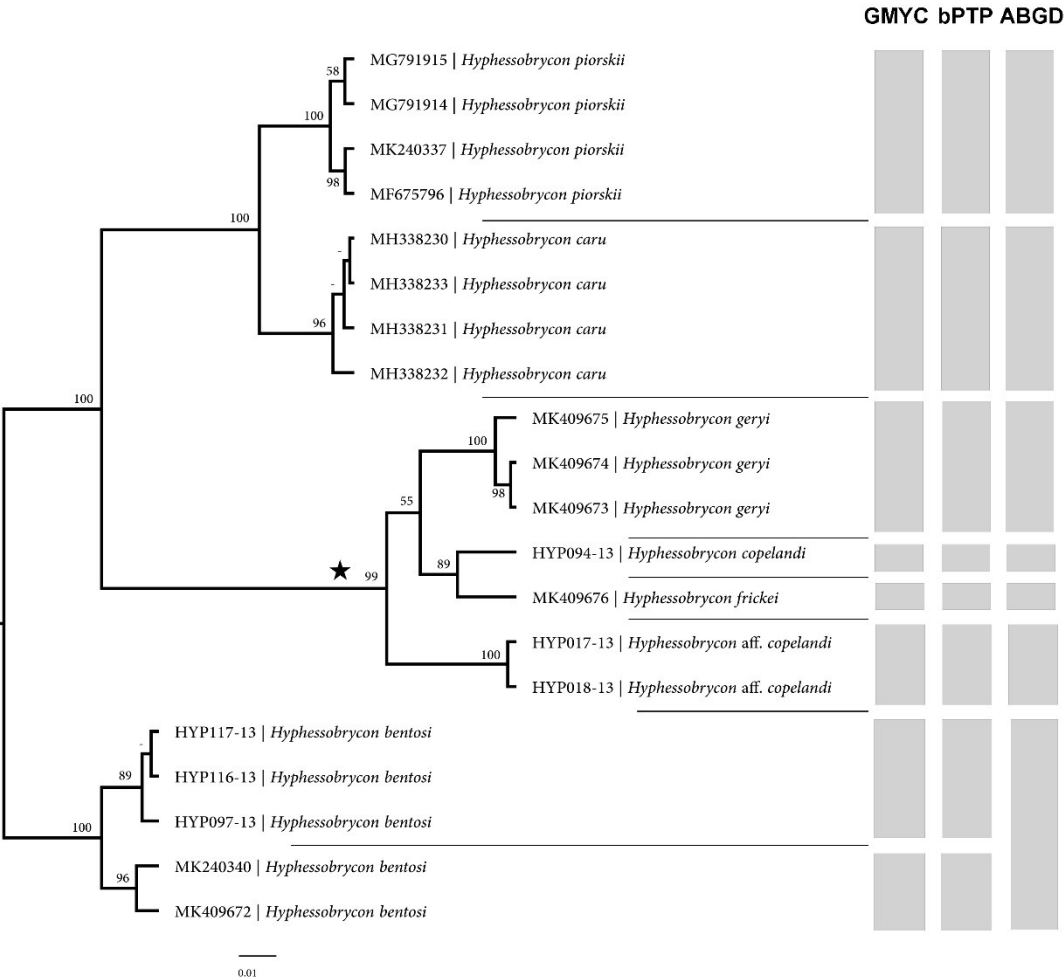


Table 1. Sampling sites, specimens and DNA sequence information included in the study.

Sequences made available by this study in bold.

Nº	Species	GenBank/BoldSystems	Catalog number
1	<i>Hyphessobrycon flammeus</i>	FUPR988-09	LBPV-40464
2	<i>Hyphessobrycon compressus</i>	FYPM054-10	CINV-NEC7411
3	<i>Hyphessobrycon compressus</i>	MXV767-15	ECO-CH P 7697
4	<i>Hyphessobrycon compressus</i>	MXV765-15	ECO-CH P 7697
5	<i>Hyphessobrycon compressus</i>	MXV766-15	ECO-CH P 7697
6	<i>Hyphessobrycon panamensis</i>	BSFFA760-07	STRI-05303
7	<i>Hyphessobrycon pyrrhonotus</i>	HYP040-13	INPA 37672-TRO10
8	<i>Hyphessobrycon pyrrhonotus</i>	HYP041-13	INPA 37672-TRO11
9	<i>Hyphessobrycon pyrrhonotus</i>	HYP157-13	JIR2003121101-6469
10	<i>Hyphessobrycon pyrrhonotus</i>	HYP158-13	JIR2003121101-6470
11	<i>Hyphessobrycon erythrostigma</i>	HYP076-13	INPA 37681-HERY1
12	<i>Hyphessobrycon erythrostigma</i>	HYP077-13	INPA37681-HERY10
13	<i>Hyphessobrycon erythrostigma</i>	HYP078-13	INPA 37681-HERY2
14	<i>Hyphessobrycon erythrostigma</i>	HYP079-13	INPA 37681-HERY3
15	<i>Hyphessobrycon socolofi</i>	HYP131-13	INPA_39530-6152
16	<i>Hyphessobrycon socolofi</i>	HYP134-13	INPA_39530-6155
17	<i>Hyphessobrycon socolofi</i>	HYP148-13	INPA 39530-BCR8
18	<i>Hyphessobrycon socolofi</i>	HYP135-13	INPA_39530-6178
19	<i>Hyphessobrycon bentosi</i>	MK409672	-
20	<i>Hyphessobrycon bentosi</i>	MK240340	CICCAA2350
21	<i>Hyphessobrycon bentosi</i>	MK240341	CICCAA2351
22	<i>Hyphessobrycon bentosi</i>	MK240339	CICCAA2349
23	<i>Hyphessobrycon bentosi</i>	HYP117-13	INPA_39527-BA2
24	<i>Hyphessobrycon bentosi</i>	HYP116-13	INPA_39527-BA1
25	<i>Hyphessobrycon bentosi</i>	HYP097-13	INPA_37684-5939
26	<i>Hyphessobrycon bentosi</i>	HYP098-13	INPA_37684-5940
27	<i>Hyphessobrycon piorskii</i>	MG791915	CICCAA1650-1
28	<i>Hyphessobrycon piorskii</i>	MG791914	CICCAA01651-1
29	<i>Hyphessobrycon piorskii</i>	MF765796	CICCAA00725-1
30	<i>Hyphessobrycon piorskii</i>	MF765797	CICCAA00726-1
31	<i>Hyphessobrycon piorskii</i>	MK240337	CICCAA2164-1
32	<i>Hyphessobrycon piorskii</i>	MK240338	CICCAA2164-4
33	<i>Hyphessobrycon caru</i>	MH338230	CICCAA00748-1
34	<i>Hyphessobrycon caru</i>	MH338231	CICCAA00749-1
35	<i>Hyphessobrycon caru</i>	MH338232	CICCAA02300-1
36	<i>Hyphessobrycon caru</i>	MH338233	CICCAA02301-1

37	<i>Hyphessobrycon micropterus</i>	BSB287-10	-
38	<i>Hyphessobrycon micropterus</i>	BSB289-10	-
39	<i>Hyphessobrycon micropterus</i>	BSB290-10	-
40	<i>Hyphessobrycon micropterus</i>	BSB291-10	-
41	<i>Hyphessobrycon simulatus</i>	GBOL1771-17	-
42	<i>Hyphessobrycon simulatus</i>	GBOL3298-18	MHNG 2757.080
43	<i>Hyphessobrycon simulatus</i>	GBOL3300-18	MHNG 2759.026
44	<i>Hyphessobrycon simulatus</i>	GBOL3301-18	MHNG 2759.026
45	<i>Hyphessobrycon simulatus</i>	GBOL3302-18	MHNG 2759.035
46	<i>Hyphessobrycon eques</i>	DSMIS077-09	CBG OL 0544
47	<i>Hyphessobrycon eques</i>	HYP070-13	INPA_37678-IC2
48	<i>Hyphessobrycon eques</i>	HYP071-13	INPA_37679-PE1
49	<i>Hyphessobrycon eques</i>	HYP072-13	INPA_37680-AL1
50	<i>Hyphessobrycon geryi</i> sp. n.	MK409673	CICCAA2047-1
51	<i>Hyphessobrycon geryi</i> sp. n.	MK409674	CICCAA2047-3
52	<i>Hyphessobrycon geryi</i> sp. n.	MK409675	CICCAA2047-6
53	<i>Hyphessobrycon frickei</i> sp. n.	MK409676	CICCAA2302-1
54	<i>Hyphessobrycon frickei</i> sp. n.	MK409677	CICCAA2302-2
55	<i>Hyphessobrycon frickei</i> sp. n.	MK409678	CICCAA2302-3
56	<i>Hyphessobrycon copelandi</i>	HYP094-13	INPA_37683-TU1
57	<i>Hyphessobrycon copelandi</i>	HYP095-13	INPA_37683-TU2
58	<i>Hyphessobrycon copelandi</i>	HYP096-13	INPA_37683-TU3
59	<i>Hyphessobrycon</i> aff. <i>copelandi</i>	HYP016-13	INPA_37666-MARA6
60	<i>Hyphessobrycon</i> aff. <i>copelandi</i>	HYP017-13	INPA_37666-MARA7
61	<i>Hyphessobrycon</i> aff. <i>copelandi</i>	HYP018-13	-
62	<i>Hyphessobrycon</i> aff. <i>copelandi</i>	HYP019-13	-
63	<i>Hyphessobrycon epicharis</i>	HYP002-13	INPA_37665-JUF1
64	<i>Hyphessobrycon epicharis</i>	HYP004-13	INPA_37665-JUF3
65	<i>Hyphessobrycon epicharis</i>	HYP005-13	INPA_37665-JUF4
66	<i>Hyphessobrycon epicharis</i>	HYP006-13	INPA_37665-JUF8
67	<i>Hyphessobrycon</i> cf. <i>sweglesi</i>	HYP026-13	INPA_37668-JAR3
68	<i>Hyphessobrycon</i> cf. <i>sweglesi</i>	HYP027-13	INPA_37668-JAR4
69	<i>Hyphessobrycon</i> cf. <i>sweglesi</i>	HYP028-13	INPA_37668-JAR5
70	<i>Hyphessobrycon</i> cf. <i>sweglesi</i>	HYP030-13	INPA_37668-JAR7
71	<i>Hyphessobrycon roseus</i>	GBOL759-15	-
72	<i>Hyphessobrycon roseus</i>	GBOL1785-17	-
73	<i>Hyphessobrycon roseus</i>	GBOL760-15	-
74	<i>Hyphessobrycon roseus</i>	GBOL3336-18	-
75	<i>Pristella maxillaris</i>	KU568982.1	-
76	<i>Pristella maxillaris</i>	KU568981.1	-
77	<i>Pristella maxillaris</i>	TZGAA025-06	I012-1.3

78	<i>Pristella maxillaris</i>	TZGAA178-06	I014-7.4
79	<i>Moenkhausia hemigrammoides</i>	HYP101-13	INPA38532-PR1
80	<i>Moenkhausia hemigrammoides</i>	HYP102-13	INPA_38532-PR2
81	<i>Moenkhausia hemigrammoides</i>	HYP103-13	INPA_38532-PR3

Table 2. Morphometric data (N=18) for the holotype and paratypes of *Hyphessobrycon frickei* sp. n. SD: Standard deviation.

	Holotype	Paratypes	Mean	SD
Standard length	18.8	14.8-19.6	17.4	-
Percentages of standard length				
Depth at dorsal-fin origin (body depth)	30.8	28.4-32.8	30.5	1.2
Snout to dorsal-fin origin	50.7	47.2-52.1	50.0	1.2
Snout to pectoral-fin origin	28.6	26.2-30.7	28.2	1.4
Snout to pelvic-fin origin	44.6	43.1-47.5	45.1	1.3
Snout to anal-fin origin	58.5	57.0-62.2	59.3	1.4
Caudal peduncle depth	9.7	8.6-11.0	10.0	0.7
Caudal peduncle length	11.4	9.1-12.1	10.4	0.9
Pectoral-fin length	20.4	17.5-22.7	19.6	1.3
Pelvic-fin length	17.1	16.0-19.3	17.3	0.9
Dorsal-fin base length	14.7	13.5-16.3	14.8	1.0
Dorsal-fin height	27.4	27.2-33.1	29.1	1.6
Anal-fin base length	33.2	30.8-35.3	33.1	1.0
Eye to dorsal-fin origin	35.6	32.5-37.3	34.4	1.1
Dorsal-fin origin to caudal-fin base	56.5	51.1-56.3	53.4	1.4
Head length	28.2	26.7-30.9	28.7	1.2
Percentages of head length				
Horizontal eye diameter	41.8	40.6-45.9	42.7	1.6
Snout length	22.8	17.5-24.0	21.6	1.9
Least interorbital width	25.7	24.1-30.6	27.4	1.7
Upper jaw length	36.5	33.2-37.6	35.3	1.6

Table 3. Morphometric data (N=53) for the holotype and paratypes of *Hyphessobrycon geryi* sp. n. SD: Standard deviation.

	Holotype	Paratypes	Mean	SD
Standard length	28.2	15.9-26.1	19.7	-
Percentages of standard length				
Depth at dorsal-fin origin (body depth)	32.4	27.7-34.4	31.5	1.2
Snout to dorsal-fin origin	51.5	49.6-53.6	51.5	0.8
Snout to pectoral-fin origin	25.7	26.4-29.7	28.2	1.1
Snout to pelvic-fin origin	42.7	44.1-47.0	45.1	0.8
Snout to anal-fin origin	57.2	53.4-63.4	58.8	1.7
Caudal peduncle depth	9.8	7.0-11.3	9.8	0.9
Caudal peduncle length	11.3	8.3-12.8	10.2	1.0
Pectoral-fin length	19.2	14.8-22.18	19.7	1.6
Pelvic-fin length	17.0	14.4-20.1	16.2	1.4
Dorsal-fin base length	13.9	11.9-15.9	14.4	0.8
Dorsal-fin height	25.5	23.5-33.0	28.1	1.8
Anal-fin base length	31.3	30.2-34.6	32.7	1.2
Eye to dorsal-fin origin	37.4	34.5-39.2	36.7	0.9
Dorsal-fin origin to caudal-fin base	54.7	50.5-56.2	53.7	1.1
Head length	27.6	26.9-29.8	29.1	0.6
Percentages of head length				
Horizontal eye diameter	36.8	36.8-46.1	41.4	2.3
Snout length	20.9	19.5-26.3	22.8	1.7
Least interorbital width	27.1	25.5-33.3	28.8	1.1
Upper jaw length	32.4	30.2-37.7	33.2	2.6

Table 4. Kimura-2 parameters pairwise genetic distances among *Hyphessobrycon* species of the “Rosy tetra” clade. Species of the *Hyphessobrycon*

copelandi clade in bold.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	<i>H. bentosi</i>															
2	<i>H. compressus</i>	0.216														
3	<i>H. cf. sweglesi</i>	0.219	0.214													
4	<i>H. roseus</i>	0.216	0.208	0.217												
5	<i>H. epicharis</i>	0.225	0.217	0.033	0.213											
6	<i>H. erythrostigma</i>	0.104	0.219	0.206	0.219	0.233										
7	<i>H. pyrrhonotus</i>	0.098	0.219	0.201	0.223	0.230	0.008									
8	<i>H. socolofi</i>	0.108	0.220	0.200	0.192	0.226	0.038	0.038								
9	<i>H. micropterus</i>	0.186	0.213	0.182	0.210	0.174	0.201	0.196	0.195							
10	<i>H. simulatus</i>	0.182	0.209	0.182	0.183	0.179	0.189	0.188	0.178	0.045						
11	<i>H. caru</i>	0.194	0.210	0.207	0.204	0.193	0.202	0.202	0.199	0.055	0.060					
12	<i>H. piorskii</i>	0.200	0.205	0.205	0.195	0.197	0.190	0.185	0.186	0.069	0.066	0.042				
13	<i>H. eques</i>	0.201	0.211	0.199	0.182	0.198	0.185	0.191	0.168	0.164	0.162	0.170	0.164			
14	<i>H. aff. copelandi</i>	0.208	0.198	0.220	0.195	0.209	0.188	0.185	0.182	0.187	0.183	0.173	0.168	0.102		
15	<i>H. geryi</i> sp. n.	0.205	0.206	0.209	0.196	0.195	0.194	0.191	0.190	0.185	0.185	0.171	0.176	0.097	0.041	
16	<i>H. frickei</i> sp. n.	0.208	0.218	0.212	0.210	0.204	0.193	0.190	0.187	0.171	0.167	0.166	0.160	0.099	0.060	0.047
17	<i>H. copelandi</i>	0.220	0.213	0.211	0.209	0.202	0.201	0.198	0.190	0.181	0.176	0.173	0.168	0.106	0.058	0.035

Box 1. List of nucleotide substitutions (synapomorphies and autapomorphies) from each lineage (species) and some crucial points of the cladogram of the Fig. 7b.

A (*Hyphessobrycon copelandi* clade)- COI 90 (C→A), COI 126 (A→G), COI 138 (C→T), COI 189 (C→T), COI 192 (T→A), COI 237 (C→T), COI 264 (T→C), COI 282 (C→T), COI 285 (C→A), COI 312 (T→C), COI 384 (C→T), COI 402 (A→G), COI 429 (A→G), COI 435 (A→G), COI 486 (T→C), COI 522 (A→G), COI 525 (C→T), COI 547 (C→T), COI 582 (T→C), COI 621 (T→C), COI 624 (A→G), COI 678 (T→C), COI 684 (A→C), COI 690 (T→C), COI 696 (A→G). **B (All the species except *H. aff. copelandi*)** - COI 162 (C→T), COI 270 (A→C), COI 276 (G→A), COI 363 (G→A), COI 681 (C→T). **C (*H. aff. copelandi*)** - COI 93 (G→A), COI 114 (G→A), COI 132 (T→C), COI 228 (A→G), COI 244 (C→T), COI 246 (G→A), COI 330 (C→T), COI 336 (C→A), COI 414 (T→C), COI 591 (A→G), COI 672 (T→C). **D (*H. geryi* sp. n.)** - COI 225 (T→C), COI 228 (A→C), COI 321 (G→A), COI 522 (G→A), COI 534 (G→A), COI 543 (A→G), COI 589 (C→T), COI 615 (G→A). **E (*H. frickei* sp. n. + *H. copelandi*)** - COI 231 (T→C), COI 315 (A→G), COI 345 (A→C), COI 375 (G→A), COI 402 (G→A), COI 405 (T→C), COI 528 (A→G), COI 558 (A→G), COI 609 (A→G). **F (*H. frickei* sp. n.)** - COI 126 (G→A), COI 141 (G→C), COI 291 (A→G), COI 300 (A→G), COI 345 (C→G), COI 366 (C→T), COI 435 (G→A), COI 510 (T→C), COI 633 (T→C), COI 657 (C→T), COI 672 (T→C). **G (*H. copelandi*)** - COI 84 (A→G), COI 246 (G→A), COI 282 (T→C), COI 321 (G→T), COI 339 (A→G), COI 375 (A→C), COI 417 (A→G), COI 429 (G→A), COI 450 (T→C), COI 504 (T→C), COI 663 (C→T).

ANEXOS

A new cryptic species of *Hyphessobrycon* Durbin, 1908 (Characiformes, Characidae) from the Eastern Amazon, revealed by integrative taxonomy

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<http://zoobank.org/E45AD907-EFD2-46B9-B056-31250BC8BFEE>

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Abstract

Hyphessobrycon caru **sp. nov.** is described based on five different and independent methods of species delimitation, making the hypothesis of this new species supported by an integrative taxonomy perspective. This new species has a restricted distribution, occurring just in the upper Pindaré river drainage, Mearim river basin, Brazil. It is a member of the rosy tetra clade, which is characterized mainly by the presence of a dark brown or black blotch on dorsal fin and absence of a midlateral stripe on the body. *Hyphessobrycon caru* **sp. nov.** is distinguished from the members of this clade mainly by the shape of its humeral spot, possessing few irregular inconspicuous vertically arranged chromatophores in the humeral region, or sometimes a very thin and inconspicuous humeral spot, and other characters related to teeth count, and color pattern. The phylogenetic position of the new species within the rosy tetra clade was based on molecular phylogenetic analysis using sequences of the mitochondrial gene cytochrome oxidase subunit 1. In addition, a new clade (here termed *Hyphessobrycon micropterus* clade) within the rosy tetra clade is proposed based on molecular data, comprising *H. caru* **sp. nov.**, *H. micropterus*, *H. piorskii*, and *H. simulatus*, and with *H. caru* **sp. nov.** and *H. piorskii* recovered as sister species. Our results suggest cryptic speciation in the rosy tetra clade and, more specifically, in the *H. micropterus* clade. We recommend the use of integrative taxonomy for future taxonomic revisions and species descriptions when dealing with species complexes and groups containing possible cryptic species.

Key Words

bPTP, DNA barcoding, rosy tetra clade, species complex, Stethaprioninae

Introduction

Hyphessobrycon Durbin, 1908 is a species-rich characid genus comprising about 160 valid species (Fricke et al. 2019). It is widely distributed along the river basins of the Neotropical region, from southern Mexico to the La Plata River basin in northeastern Argentina (Carvalho and Malabarba 2015; García-Alzate et al. 2017; Guimarães et al. 2018). The genus was first proposed as a subgenus of *Hemigrammus* Gill, 1858 by Durbin in Eigenmann (1908), differing from the latter only by the absence of scales covering the caudal-fin. *Hyphessobrycon* was reviewed by Eigenmann (1918, 1921) in a work which still constitutes the most comprehensive revisionary studies on the genus. The large number of species included within *Hyphessobrycon* and the poor knowledge of the alpha and beta-taxonomy of species and species groups are among the major challenges for a more comprehensive taxonomic study and phylogenetic analyses of the genus. It is widely known that *Hyphessobrycon* does not constitute a monophyletic group (Weitzman and Palmer 1997a; Mirande 2010, 2018; Oliveira et al. 2011; Carvalho and Malabarba 2015; Carvalho et al. 2017; Moreira and Lima 2017; Betancur-R. et al. 2018; Guimarães et al. 2018). Nevertheless, groups of species have been proposed based primarily on similarities of color pattern and other external features (e.g. Weitzman and Palmer 1997a; García-Alzate et al. 2008; Moreira and Lima 2017). Some of them are probably merely artificial operational assemblages to aid species identification, whereas others represent potential monophyletic groups, delimited by exclusive character states (e.g. Castro-Paz et al. 2014; Carvalho and Malabarba 2015; Guimarães et al. 2018).

Several genetic studies focusing on characoid fishes, such as *Astyanax* Baird & Girard, 1854 (e.g. Ornelas-Garcia et al. 2008), *Caenotropus* Günther, 1864 (e.g. Melo et al. 2014), *Chilodus* Müller & Troschel, 1844 (e.g. Melo et al. 2014), *Curimatopsis* Steindachner, 1876 (e.g. Melo et al. 2016a), *Gymnocorymbus* Eigenmann, 1908 (e.g. Benine et al. 2015), *Hyphessobrycon* (e.g. Castro-Paz et al. 2014; Guimarães et al. 2018), *Piabina* Reinhardt, 1867 (e.g. Pereira et al. 2011), *Prochilodus* Agassiz, 1829 (e.g. Melo et al. 2016b), *Nannostomus* Günther, 1872 (e.g. Benzaquem et al. 2015) and *Tetragonopterus* Bleeker, 1863 (e.g. Melo et al. 2016c) have evidenced that some species may exhibit large discontinuities in their geographic distribution patterns, with high genetic divergences, but little morphological variability among geographically isolated lineages. These results suggest that these groups may represent species complexes or cryptic species, that is, they might even including morphologically quite similar or undistinguishable species that are hidden and erroneously classified (Brown et al. 1995; Bickford et al. 2006; Adams et al. 2014; Souza et al. 2018). Studies relying solely on morphology may be inadequate in recognizing species within groups including cryptic species (Guim-

arães et al. 2018). Integrative studies, using more than one criteria, such as character-based, tree-based, genetic distance and coalescent-based approaches, especially including molecular data, are useful and powerful for the recognition of hidden and/or possible new species in such species complexes (Sytsma and Schaal 1985; Bickford et al. 2006; Goldstein and Desalle 2010; Padial et al. 2010; Adams et al. 2014; Costa-Silva et al. 2015; Souza et al. 2018; Ottoni et al. 2019).

In this context of integrative taxonomy, the present study aims to investigate the diversity within the rosy tetra clade sensu Weitzman and Palmer (1997a). This clade comprises around 30 species, including some species of *Hyphessobrycon* and other allied species, that are appreciated as aquarium fishes due to their attractive color patterns (e.g. Weitzman and Palmer 1997a, 1997b, 1997c, 1997d; Zarske 2008; Hein 2009; Guimarães et al. 2018).

This group has had its composition and name changed over the last decades, and a detailed taxonomic history is presented by Weitzman and Palmer (1997a). Two previous papers (e.g. Castro-Paz et al. 2014; Guimarães et al. 2018) applied molecular approaches to investigate the diversity of rosy tetra clade, and they suggested that its taxonomic resolution should be better investigated as it could include cryptic species or valid species which may have been synonymized. A new species of *Hyphessobrycon* and member of the rosy tetra clade is described from the upper Pindaré river drainage, Mearim river basin, a coastal river basin of the Eastern Amazon region, Brazil, based on both morphology and molecular data. Furthermore, a new clade, within the rosy tetra clade, is proposed based on the phylogenetic tree topology presented.

Materials and methods

Taxa sampling, specimens collection, and preservation

Individuals collected for this study were euthanized with a buffered solution of MS-222 at a concentration of 250 mg L⁻¹ for a period of 10 min or more until opercular movements completely ceased. Specimens selected for morphological analysis were fixed in formalin and left for 10 days, after which they were preserved in 70% ethanol. Molecular data were obtained from specimens that were euthanized, fixed, and preserved in absolute ethanol.

Specimens for morphological analysis are listed in type and comparative material lists. Specimens for molecular approaches are listed in Table 1. We also retrieved sequences from other species of *Hyphessobrycon* and allied species for a comparative analysis from the Barcode of Life Database (BOLD) and the National Center for Biotechnology Information (NCBI) databases (Table 1).

Table 1. List of species, specimens and their respective catalogue numbers, Region/state/country, and BOLD Systems and GenBank sequence accession numbers. Sequences available in the current study are in Bold.

N°	Species	Catalogue number	Region/state/country	Accession no.
1	<i>Hyphessobrycon erythrostigma</i>	INPA 37681-HERY1	Tabatinga/Amazonas/Brazil	HYP076-13
2	<i>Hyphessobrycon erythrostigma</i>	INPA 37681-HERY10	Tabatinga/Amazonas/Brazil	HYP077-13
3	<i>Hyphessobrycon erythrostigma</i>	INPA 37681-HERY2	Tabatinga/Amazonas/Brazil	HYP078-13
4	<i>Hyphessobrycon erythrostigma</i>	INPA 37681-HERY3	Tabatinga/Amazonas/Brazil	HYP079-13
5	<i>Hyphessobrycon pyrrhonotus</i>	INPA 37672-TRO10	Santa Isabel do Rio Negro/Amazonas/Brazil	HYP040-13
6	<i>Hyphessobrycon pyrrhonotus</i>	INPA 37672-TRO11	Santa Isabel do Rio Negro/Amazonas/Brazil	HYP041-13
7	<i>Hyphessobrycon pyrrhonotus</i>	–	Barcelos/Amazonas/Brazil	HYP157-13
8	<i>Hyphessobrycon pyrrhonotus</i>	–	Barcelos/Amazonas/Brazil	HYP158-13
9	<i>Hyphessobrycon socolofi</i>	INPA_39530-6152	Barcelos/Amazonas/Brazil	HYP131-13
10	<i>Hyphessobrycon socolofi</i>	INPA_39530-6155	Barcelos/Amazonas/Brazil	HYP134-13
11	<i>Hyphessobrycon socolofi</i>	INPA_39530-6178	Barcelos/Amazonas/Brazil	HYP135-13
12	<i>Hyphessobrycon socolofi</i>	INPA 39530-BCR8	Barcelos/Amazonas/Brazil	HYP148-13
13	<i>Hyphessobrycon copelandi</i>	INPA_37683-TU1	Tabatinga/Amazonas/Brazil	HYP094-13
14	<i>Hyphessobrycon copelandi</i>	INPA_37683-TU2	Tabatinga/Amazonas/Brazil	HYP095-13
15	<i>Hyphessobrycon copelandi</i>	INPA_37683-TU3	Tabatinga/Amazonas/Brazil	HYP096-13
16	<i>Hyphessobrycon eques</i>	INPA_37678-IC2	Santarém/Pará/Brazil	HYP070-13
17	<i>Hyphessobrycon eques</i>	INPA_37679-PE1	Macapá/Amapá/Brazil	HYP071-13
18	<i>Hyphessobrycon eques</i>	INPA_37680-AL1	Parintins/Amazonas/Brazil	HYP072-13
19	<i>Hyphessobrycon eques</i>	OL-0544	Bonito/Mato Grosso do Sul/Brazil	DSMIS077-09
20	<i>Hyphessobrycon epicharis</i>	INPA_37665-JUF1	São Gabriel da Cachoeira/Amazonas/Brazil	HYP002-13
21	<i>Hyphessobrycon epicharis</i>	INPA_37665-JUF3	São Gabriel da Cachoeira/Amazonas/Brazil	HYP004-13
22	<i>Hyphessobrycon epicharis</i>	INPA_37665-JUF4	São Gabriel da Cachoeira/Amazonas/Brazil	HYP005-13
23	<i>Hyphessobrycon epicharis</i>	INPA_37665-JUF8	São Gabriel da Cachoeira/Amazonas/Brazil	HYP006-13
24	<i>Hyphessobrycon compressus</i>	CINV-NEC7411	Flores Magon/Campeche/México	FYPM054-10
25	<i>Hyphessobrycon compressus</i>	ECOC	Hatfield/Belize/Belize	MXV765-15
26	<i>Hyphessobrycon compressus</i>	ECOC	Hatfield/Belize/Belize	MXV766-15
27	<i>Hyphessobrycon compressus</i>	ECOC	Hatfield/Belize/Belize	MXV767-15
28	<i>Hyphessobrycon bentosi</i>	INPA_37684-5939	Barcelos/Amazonas/Brazil	HYP097-13
29	<i>Hyphessobrycon bentosi</i>	INPA_37684-5940	Barcelos/Amazonas/Brazil	HYP098-13
30	<i>Hyphessobrycon bentosi</i>	INPA_39527-BA1	–	HYP116-13
31	<i>Hyphessobrycon bentosi</i>	INPA_39527-BA2	–	HYP117-13
32	<i>Hyphessobrycon bentosi</i>	CICCAA02349	Santarém/Pará/Brazil	MK240339
33	<i>Hyphessobrycon bentosi</i>	CICCAA02350	Santarém/Pará/Brazil	MK240340
34	<i>Hyphessobrycon bentosi</i>	CICCAA02351	Santarém/Pará/Brazil	MK240341
35	<i>Hyphessobrycon simulatus</i>	MHNG 2743.087	Pisimmoengo/Commewijne/Suriname	GBOL761-15
36	<i>Hyphessobrycon simulatus</i>	MHNG 2743.087	Pisimmoengo/Commewijne/Suriname	GBOL762-15
37	<i>Hyphessobrycon simulatus</i>	–	Sinnamary/Cayenne/French Guiana	GBOL1771-17
38	<i>Hyphessobrycon simulatus</i>	MHNG 2735.007	Sinnamary/Cayenne/French Guiana	GBOL3296-18
39	<i>Hyphessobrycon simulatus</i>	MHNG 2757.080	Kourou/Cayenne/French Guiana	GBOL3298-18
40	<i>Hyphessobrycon simulatus</i>	MHNG 2759.026	Kaw/Cayenne/French Guiana	GBOL3300-18
41	<i>Hyphessobrycon simulatus</i>	MHNG 2759.026	Kaw/Cayenne/French Guiana	GBOL3301-18
42	<i>Hyphessobrycon simulatus</i>	MHNG 2759.035	Régina/ Cayenne/French Guiana	GBOL3302-18
43	<i>Hyphessobrycon cf. sweglesi</i>	INPA_37668-JAR3	São Gabriel da Cachoeira/Amazonas/Brazil	HYP026-13
44	<i>Hyphessobrycon cf. sweglesi</i>	INPA_37668-JAR4	São Gabriel da Cachoeira/Amazonas/Brazil	HYP027-13
45	<i>Hyphessobrycon cf. sweglesi</i>	INPA_37668-JAR5	São Gabriel da Cachoeira/Amazonas/Brazil	HYP028-13
46	<i>Hyphessobrycon cf. sweglesi</i>	INPA_37668-JAR7	São Gabriel da Cachoeira/Amazonas/Brazil	HYP030-13
47	<i>Hyphessobrycon micropterus</i>	–	Várzea da Palma/Minas Gerais/Brazil	BSB287-10
48	<i>Hyphessobrycon micropterus</i>	–	Várzea da Palma/Minas Gerais/Brazil	BSB288-10
49	<i>Hyphessobrycon micropterus</i>	–	Várzea da Palma/Minas Gerais/Brazil	BSB289-10
50	<i>Hyphessobrycon micropterus</i>	–	Várzea da Palma/Minas Gerais/Brazil	BSB290-10
51	<i>Hyphessobrycon piorskii</i>	CICCAA00725-1	Chapadinha/Maranhão/Brazil	MF765796
52	<i>Hyphessobrycon piorskii</i>	CICCAA00726-1	Chapadinha/Maranhão/Brazil	MF765797
53	<i>Hyphessobrycon piorskii</i>	CICCAA01650-1	Barreirinhas/Maranhão/Brazil	MG791915
54	<i>Hyphessobrycon piorskii</i>	CICCAA01651-1	Barreirinhas/Maranhão/Brazil	MG791914
55	<i>Hyphessobrycon piorskii</i>	CICCAA02164-1	Codó/Maranhão/Brazil	MK240337
56	<i>Hyphessobrycon piorskii</i>	CICCAA02164-4	Codó/Maranhão/Brazil	MK240338
57	<i>Hyphessobrycon caru</i>	CICCAA00748-1	Buritcupu/Maranhão/Brazil	MH338230
58	<i>Hyphessobrycon caru</i>	CICCAA00749-1	Buritcupu/Maranhão/Brazil	MH338231
59	<i>Hyphessobrycon caru</i>	CICCAA02300-1	Buritcupu/Maranhão/Brazil	MH338232
60	<i>Hyphessobrycon caru</i>	CICCAA02301-1	Buritcupu/Maranhão/Brazil	MH338233
61	<i>Pristella maxillaris</i>	–	–	KU568982.1
62	<i>Pristella maxillaris</i>	–	–	KU568981.1
63	<i>Pristella maxillaris</i>	–	Marlborough/Pomeroon-Supenaam/Guyana	TZGAA025-06
64	<i>Pristella maxillaris</i>	–	Santa Cruz/Barima-Waini/Guyana	TZGAA178-06
65	<i>Moenkhausia hemigrammoides</i>	INPA38532-PR1	Guyana	HYP101-13
66	<i>Moenkhausia hemigrammoides</i>	INPA_38532-PR2	Guyana	HYP102-13
67	<i>Moenkhausia hemigrammoides</i>	INPA_38532-PR3	Guyana	HYP103-13
68	<i>Hyphessobrycon panamensis</i>	STRI-05303	Cocle/Panama	BSFFA760-07
69	<i>Hyphessobrycon flammeus</i>	LBPV-40464	Biritiba-Mirim/São Paulo/Brazil	FUPR988-09

Morphological analysis

Measurements and counts were made according to Fink and Weitzman (1974), with exception of the scale rows below lateral line, which were counted to the insertion of pelvic-fin. Vertical scale rows between the dorsal-fin origin and lateral line do not include the scale of the median predorsal series situated just anterior to the first dorsal-fin ray. Counts of supraneurals, vertebrae, procurent caudal-fin rays, unbranched dorsal and anal-fin rays, branchiostegal rays, gill-rakers, premaxillary, maxillary, and dentary teeth were taken only from cleared and stained paratypes (C&S), prepared according to Taylor and Van Dyke (1985). The four modified vertebrae that constitute the Weberian apparatus were not included in the vertebrae counts and the fused PU1 + U1 was considered as a single element. Osteological nomenclature follows Weitzman (1962). Institutional abbreviations follow Fricke and Eschmeyer (2019), with addition of LIOP.UFAM Coleção Ictiológica do Laboratório de Ictiologia e Ordenamento Pesqueiro do Vale do Rio Madeira da Universidade Federal do Amazonas.

DNA extraction, amplification, and sequencing

DNA was extracted from fin clips using Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's protocol. Fragments of the cytochrome c oxidase subunit 1 gene (hereafter COI) from mitochondrial DNA were amplified, using the universal primers designed by Ward et al. (2005) for fish. Polymerase chain reactions (PCR) comprised a total volume of 15 µl containing 1× Polymerase buffer, 1.5 mM MgCl₂, 200 µM dNTP, 0.2 µM of each primer, 1U of Taq Polymerase (Invitrogen), 100 ng of DNA template, and ultrapure water. The PCR cycles were as follows: 2 min at 94 °C, followed by 35 cycles of 94 °C for 30s, 54 °C for 30s, and 72 °C for 1 min, and 10 min at 72 °C. Amplicons were purified using Illustra GFX PCR DNA and Gel Purification Kit (GE Healthcare Systems) and sequenced using the forward primer by an outsourced sequencing service at the University of São Paulo, using BigDye Terminator kit 3.1 Cycle Sequencing kit in ABI 3730 DNA Analyser (Applied Biosystems).

Data partition, evolution models, and alignment

The dataset included the following gene: COI (680 Base pairs, BP). Sequences were aligned using ClustalW (Chenna et al. 2003). The DNA sequences were translated into amino acids residues to test for the absence of premature stop codons or indels using the program MEGA 7 (Kumar et al. 2016). In the alignment, gaps were coded with a dash (–) and missing data with a question mark (?), but during analyses, both were treated as missing data. Measure Substitution Saturation tests were

performed in DAMBE5 (Xia 2013) according to the algorithm proposed by Xia et al. (2003). The best-fit evolutionary model (GTR+G) was calculated, using the corrected Akaike Information Criterion (AICc) determined by the jModelTest 2.1.7 (Darriba et al. 2012).

Species concept, species delimitation, and diagnoses

The unified species concept is herein adopted by expressing the conceptual definition shared by all traditional species concepts, “species are (segments of) separately evolving metapopulation lineages”, disentangling operational criterion elements to delimit taxa from species concepts (de Queiroz 2005, 2007). According to this concept, species are treated as hypothetical units and could be tested by the application of distinct criteria (species delimitation methods) (de Queiroz 2005, 2007). It allows for any criteria to separately provide evidence about species limits and identities, independently from other criteria (de Queiroz 2005, 2007). However, evidence corroborated from multiple operational criteria is considered to produce stronger support for hypotheses of lineage separation (de Queiroz 2007; Goldstein and Desalle 2010), a practice called “integrative taxonomy” (Dayrat 2005; Goldstein and Desalle 2010; Padial et al. 2010).

Five distinct and independent operational criteria for species delimitation, based on morphological and molecular data, were implemented here: Population Aggregation Analysis (Davis and Nixon 1992) (hereafter PAA); DNA barcoding, as proposed by Hebert et al. (2003a, 2003b, 2004 a, 2004b) (hereafter DBC); a tree-based method as proposed by Wiens and Penkrot (2002) (hereafter WP, following Sites and Marshall 2003); a character-based DNA barcoding as proposed by Desalle et al. (2005) (hereafter CBB); and a coalescent species delimitation method termed the Bayesian implementation of the Poisson tree processes (hereafter bPTP, following Zhang et al. 2013). All species delimitation methods here adopted, except PAA, were performed on cytochrome c oxidase subunit 1 (COI) sequences, as it is a mitochondrial gene with fast evolutionary rate, suitable for single locus species delimitation approaches (Avice 2000).

Population aggregation analysis (PAA)

The PAA (Davis and Nixon 1992) is a character-based method, in which species are delimited by unique combination of morphological character states occurring in one or more populations (Costa et al. 2014). The morphological data was based on both examined material and literature (e.g. Steindachner 1882; Meek 1904; Eigenmann, 1908; Durbin 1909; Eigenmann 1915; Ahl 1937; Fowler 1943; Géry 1960, 1961, 1964, 1977; Géry and Uj 1987; Burgess 1993; Planquette et al. 1996; Weitzman and

Palmer 1997a, 1997b, 1997c, 1997d; Zarske 2008; Hein 2009; Lima et al. 2013; Zarske 2014; Carvalho and Malabarba 2015; Carvalho et al. 2017; Guimarães et al. 2018).

Traditional DNA barcoding (DBC) and Phylogenetic analysis

We used the Kimura-2-parameters model (K2P) (Kimura 1980) to estimate the pairwise genetic distances between species in MEGA 7 software (Kumar et al. 2016). We used DnaSP v. 6 (Rozas et al. 2003) to estimate the number of variable sites and haplotypes. A Bayesian inference-based phylogenetic (BI) tree was estimated in MrBayes (Huelsenbeck and Ronquist 2001) plugin in Geneious 9.0.5 to reconstruct the evolutionary relationships among terminals using General Time Reversible (GTR+G) as evolutionary model. Bayesian tree inference was based in a chain length of 10 million, a burn-in length of 500,000 generations subsampling trees every 10,000 generations. We used a sequence of *Hyphessobrycon flammeus* Myers, 1924 as outgroup.

Wiens and Penkrot analysis (WP)

WP is based on the direct inspection of haplotype trees generated from the phylogenetic analysis having as terminals at least two individuals (haplotypes) of each focal species. In this method, the term “exclusive” is used instead of monophyletic, as the term monophyly is considered inapplicable below the species level (Wiens and Penkrot 2002). Clustered haplotypes with concordant geographic distribution forming mutual and well supported clades (exclusive lineages) are considered strong evidence for species discrimination (absence of gene flow with other lineages). When haplotypes from the same locality fail to cluster together, there is potential evidence for gene flow with other populations (Wiens and Penkrot 2002). Statistical support for clades is assessed by the posterior probability, considered as significant at values about 0.95 or higher (Alfaro and Holder 2006). When only one haplotype (specimen) from one putative population was available, the species delimitation was based on the exclusivity of the sister clade of this single haplotype, supported by significant values, allowing us to perform the test in populations with only one haplotype (Wiens and Penkrot 2002). In addition, the method allows recognition of nonexclusive lineages as species since their sister clades are exclusive and supported by significant values (Wiens and Penkrot 2002).

Character-based DNA barcoding (CBB)

The CBB is similar to the population aggregation analysis proposed by Davis and Nixon (1992), but directed to nucleotides as an alternative method for diagnosing taxa through DNA barcodes, as the original method is based on subjective cut-off distance measures to species

designation (Hebert et al. 2003a, 2003b, 2004a, 2004b). This method delimits species based on a unique combination of nucleotides within a site shared by individuals of the same population or group of populations. In addition, species were diagnosed by nucleotide substitutions following Costa et al. (2014). Optimization of nucleotide substitutions among lineages of the *Hyphessobrycon micropterus* clade were obtained from the Maximum Parsimony topology, using TNT 1.5 (Goloboff and Catalano 2016). Maximum Parsimony analysis (MP) was obtained with the following parameters: traditional search, tree bisection reconnection branch swapping (TBR), 1 random seed, setting random taxon-addition replicates to 1,000, multi-trees in effect, collapsing branches of zero length, characters equally weighted, and 10,000 trees saved per replication. MP tree branch support was given by bootstrap analysis (Felsenstein 1985), using a heuristic search with 1,000 replicates and the same settings used in the MP search, saving a maximum of 1,000 trees in each random taxon-addition replicate. The analysis was rooted on *Hyphessobrycon flammeus* Myers, 1924. Each nucleotide substitution is represented by its relative numeric position determined through sequence alignment with the complete mitochondrial genome of *Astyanax paranae* Eigenmann 1914 (KX609386.1:5503-7062 – mitochondrion complete genome), followed by the specific nucleotide substitution in parentheses. The results of this analysis are presented in Suppl. material 1: Box 1 and molecular diagnosis section.

Bayesian implementation of the poisson tree processes (bPTP)

The bPTP is a coalescent phylogeny-based species delimitation method aimed at delimiting species based on single locus molecular data (Zhang et al. 2013). An advantage of bPTP is that it does not need an ultrametric calibration like other coalescent approaches, avoiding errors and computer intensive processes (Zhang et al. 2013). The method relies on the number of substitutions between haplotypes and assumes that more molecular variability is expected between species than within a species (Zhang et al. 2013). In our analysis the dataset was reduced to include only unique haplotypes from the species of the *H. micropterus* clade. Outgroups were restricted to *Hyphessobrycon bentosi* Durbin, 1908 and *Hyphessobrycon copelandi* Durbin 1908. Sequences were aligned using ClustalW (Chenna et al. 2003). The best-fit evolutionary model (GTR+G) for the reduced dataset was calculated using the corrected Akaike Information Criterion (AICc) determined by the jModelTest 2.1.7 (Darriba et al. 2012). The input phylogenetic tree was performed in MrBayes 3.2.6 (Ronquist et al. 2012), with the following parameters: independent runs of two Markov chain Monte Carlo (MCMC) runs of four chains each for 3 million generations and sampling frequency of 1,000. The bPTP analysis was performed in the Exelixis Lab’s web server <http://species.h-its.org/ptp/>, following the default parameters except for a 20% burn in.

Results

Hyphessobrycon caru sp. nov.

<http://zoobank.org/3BC35EBB-E138-4E24-A06E-DF985F015ED5>

Figures 1, 2a; Table 2

Holotype. CICCAA 02286, 22.2 mm SL, Brazil, Maranhão state, Buriticupu municipality, Buritizinho river, Pindaré river drainage, Mearim river basin, 04°22'52"S, 46°30'35"W, 24 Jan. 2017, Guimarães E. C., Brito P. S.

Paratypes. All from Brazil, Maranhão state: CICCAA 00706, 37, 15.9–25.4 mm SL; CICCAA 0709, 12 C&S, 15.1–20.6 mm SL; LIOP.UFAM 1009, 1, 16.2 mm SL collected with holotype. CICCAA00707, 3, 17.2–22.1 mm SL, Buriticupu municipality, Buritizinho river, Pindaré river drainage, Mearim river basin, 4°25'45"S, 46°29'41"W, 24 Jan. 2017, Guimarães E. C., Brito P. S. CICCAA00708, 2, 19.9–21.6 mm SL, Buriticupu municipality, Buritizinho river, Pindaré river drainage, Mearim river basin, 04°19'45"S, 46°29'46"W, 24 Jan. 2017, Guimarães E. C., Brito P. S. UFRJ11745, 1, 22.4 mm SL, Buriticupu municipality, Buritizinho river, Pindaré river drainage, Mearim river basin, 04°19'45"S, 46°29'46"W, 24 Jan. 2017, Guimarães E. C., Brito P. S.

Diagnosis (PAA). The new species *Hyphessobrycon caru* sp. nov. differs from most of its congeners, except members of the rosy tetra clade, by the presence of a dark brown or black blotch on dorsal-fin (vs absence) and absence of a midlateral stripe on the body (vs presence).

The new species differs from most of its congeners in the rosy tetra clade by possessing few irregular inconspicuous vertically arranged chromatophores in the humeral region, or sometimes a very thin and inconspicuous humeral spot (Fig. 2a) [vs inconspicuous vertically elongated humeral spot in *H. hasemani* Fowler, 1913, *H. piorskii* Guimarães, De Brito, Feitosa, Carvalho-Costa, Ottoni, 2018 (Fig. 2b); approximately rounded humeral spot in *H. erythrostigma* (Fowler, 1943), *H. jackrobertsi* Zarske, 2014, *H. minor* Durbin, 1909, *H. pando* Hein, 2009, *H. paepkei* Zarske, 2014, *H. pyrrhonotus* Burgess, 1993, *H. roseus* (Géry, 1960), *H. socolofi* Weitzman, 1977, and *H. sweglesi* (Géry, 1961) (Fig. 2c); humeral spot horizontally or posteriorly elongated in *H. epicharis* Weitzman & Palmer, 1997, *H. khardinae* Zarske, 2008, and *H. wernerii* Géry & Uj, 1987 (Fig. 2d); conspicuous humeral spot at least on males in *H. copelandi* Durbin, 1908, *H. eques* (Steindachner, 1882), *H. haraldschultzi* Travassos, 1960, *H. micropterus* (Eigenmann, 1915), *H. megalopterus* (Eigenmann, 1915), *H. simulatus* (Géry, 1960) and *H. takasei* Géry, 1964 (Fig. 2e); and absence of humeral spot in *H. compressus* (Meek, 1904), *H. dorsalis* Zarske, 2014, *H. georgettae* Géry, 1961, *H. pulchripinnis* Ahl, 1937, and *H. rosaceus* Durbin, 1909 (Fig. 2f)].

Furthermore, the new species differs from *H. bentosi* Durbin, 1908, *H. erythrostigma*, *H. pyrrhonotus*, *H. rosaceus*, and *H. socolofi* by presenting only one tooth in

the outer row of premaxillary, and this unique tooth just slightly displaced from inner row [vs two or more teeth, displaced from the inner row]; from *H. hasemani* and *H. micropterus* by the dorsal-fin spot located approximately at the middle of the fin's depth, not reaching its tip (vs spot located approximately at the middle of the fin's depth, reaching its tip in adults); from *H. hasemani* by presenting tri to unicuspid teeth in the inner row of premaxillary and dentary (vs tricuspid or pentacuspid teeth); from *H. piorskii* by having the anal-fin profile usually nearly straight (vs anal-fin profile usually falcate). In addition, *H. caru* sp. nov. is easily distinguished from *Pristella maxillaris* (Ulrey, 1894), *Moenkhausia hemigrammoides* Géry, 1965, and *Hemigrammus unilineatus* (Gill, 1858) by the absence of a black oblique stripe or band on the anterior portion of the anal-fin (Fig. 1) (vs presence).

Description. Morphometric data of holotype and paratypes are presented in Table 2. Body small (with maximum SL of 25.4 mm), compressed, moderately deep, greatest body depth slightly anterior to dorsal-fin base. Lateral body profile straight and downward directed from the end of dorsal-fin to adipose-fin, straight or slightly convex between later point and origin of dorsal most procurent caudal-fin ray. Dorsal profile of head convex from upper lip to vertical through eye; predorsal profile of body roughly straight, dorsal-fin base slightly convex, posteroventrally inclined; ventral profile of head convex from lower jaw to pelvic-fin origin. Ventral profile of body straight or slightly convex from pelvic-fin origin to anal-fin origin; straight and posterodorsally slanted along anal-fin base; and slightly concave on caudal peduncle. Jaws equal, mouth terminal, anteroventral end of dentary protruding. Maxilla reaching vertical to anterior margin of pupil.

Table 2. Morphometric data ($N = 45$) of *Hyphessobrycon caru* sp. nov. SD: Standard deviation.

	Holotype	Paratypes	Mean	SD
Standard length	22.2	14.8–25.4	18.9	–
Percentages of standard length				
Depth at dorsal-fin origin (body depth)	37.3	33.1–38.5	35.2	1.1
Snout to dorsal-fin origin	53.7	49.4–55.0	51.7	1.2
Snout to pectoral-fin origin	29.5	28.2–32.3	29.9	1.0
Snout to pelvic-fin origin	46.0	43.6–48.8	45.6	1.0
Snout to anal-fin origin	62.5	58.5–64.0	61.0	1.3
Caudal peduncle depth	12.3	8.5–12.3	10.3	0.8
Caudal peduncle length	11.7	9.5–12.7	11.2	0.8
Pectoral-fin length	23.2	16.5–23.7	19.6	1.9
Pelvic-fin length	20.6	14.1–20.5	17.4	1.4
Dorsal-fin base length	15.2	12.9–15.7	14.3	0.8
Dorsal-fin height	32.2	27.9–34.1	30.8	1.5
Anal-fin base length	32.4	26.4–32.7	29.6	1.3
Eye to dorsal-fin origin	37.5	34.4–38.8	37.3	0.9
Dorsal-fin origin to caudal-fin base	55.1	50.6–56.1	53.4	1.1
Head length	29.8	27.4–31.1	29.3	1.0
Percentages of head length				
Horizontal eye diameter	39.2	35.4–43.6	39.2	1.7
Snout length	24.4	17.3–24.3	21.5	1.8
Least interorbital width	29.1	22.4–30.7	27.2	1.8
Upper jaw length	37.8	33.1–42.5	37.4	2.1



Figure 1. *Hyphessobrycon caru* sp. nov., CICCAA 02286, holotype, 22.2 mm SL; Brazil: Maranhão state: Buritizinho River, Pindaré river drainage, Mearim river basin.

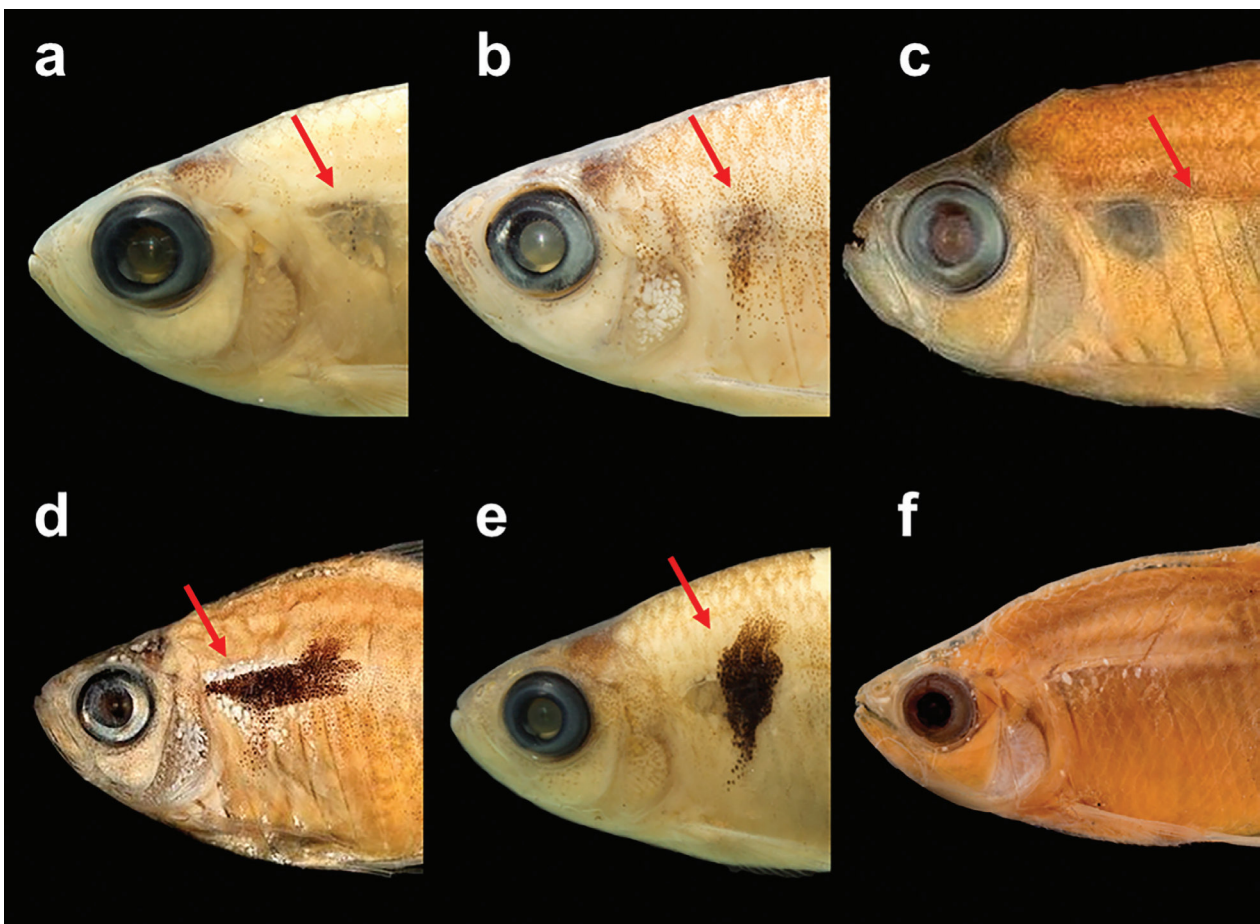


Figure 2. Humeral spot of: **a** *Hyphessobrycon caru*, holotype, CICCAA 02286 **b** *H. piorskii*, holotype, CICCAA 00695 **c** *H. pyr-rhonotus*, holotype, MZUSP 45714 **d** *H. weneri*, holotype, MZUSP 42365 **e** *H. eques*, CICCAA 00300 **f** *H. compressus*, paratype, MHNG 2181.076.

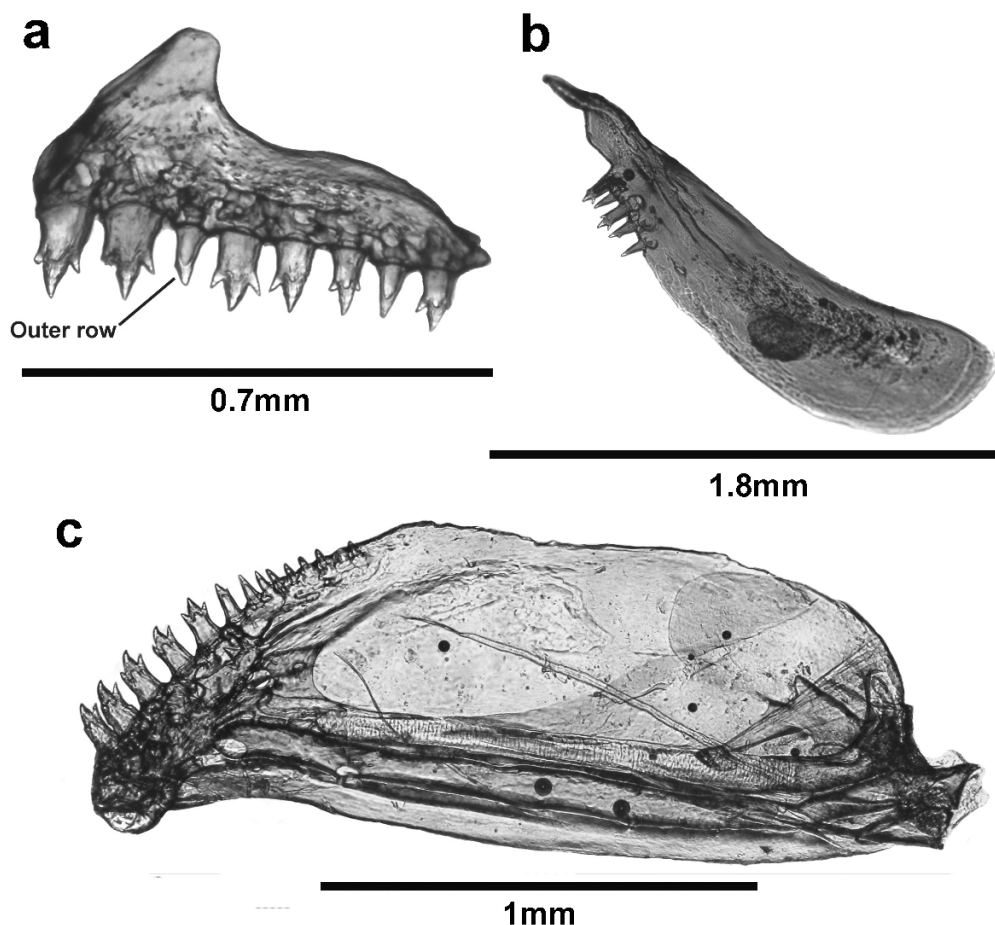


Figure 3. *Hyphessobrycon caru* sp. nov., jaw suspensory, CICCAA 00697, paratype, 19.3 mm SL: premaxillary (a), maxillary (b), and dentary (c).

Premaxillary teeth in two rows. Outer row with one unicuspid or tricuspid tooth, just slightly displaced from inner row; inner row with 6(5), 7(6), or 8(1) tricuspid teeth and one unicuspid tooth. Maxilla with 3(2) tricuspid teeth and two unicuspid teeth, 4(3) tricuspid teeth and two unicuspid teeth or 5(7) tricuspid teeth. Dentary with five (10) or six (1) larger tricuspid teeth followed by one smaller tricuspid teeth 5(2), 6(2), 7(3), and 8(5) smaller unicuspid teeth (Fig. 3).

Scales cycloid, three to eight radii strongly marked, circuli well-marked anteriorly, weakly marked posteriorly; lateral line incompletely pored, with 5(1), 6(2), 7(24), 8(14), or 9(4) perforated scales. Longitudinal scales series including lateral-line scales 31(1), 32(7), 33(14), 34(13), 35(3), or 36(7). Longitudinal scales rows between dorsal-fin origin and lateral line 5(3), 6(32), or 7(10). Horizontal scale rows between lateral line and pelvic-fin origin 4(43) or 5(2). Scales in median series between tip of supraoccipital spine and dorsal-fin origin 10(9), 11(12), 12(21), or 13(3). Circumpeduncular scales 11(6), 12(35), 13(2), or 14(2).

Dorsal-fin origin at midbody. Base of last dorsal-fin ray at vertical through first third of anal-fin. Dorsal-fin rays ii + 9(48), iii + 9(5), ii + 10(4). First dorsal-fin pterygiophore main body located behind neural spine of 4th

vertebrae. Adipose-fin present. Anal-fin origin aligned with vertical line through middle of dorsal-fin, between 6th and 8th dorsal-fin rays base. Anteriormost anal-fin pterygiophore inserting posterior to haemal spine of 11th vertebrae. First anal-fin ray in vertical through the middle of dorsal-fin (with about 7th or 8th ray base). Anal-fin iii + 22(10) or iii + 23(47); anal-fin origin aligned with vertical line through middle of dorsal-fin (between base of 6th and 8th dorsal-fin rays); Anal-fin profile nearly straight; Anal-fin rays with a sexually dimorphic pattern, which is absent in females, described below. Pectoral-fin rays 12(57) total rays. Tip of pectoral-fin surpassing pelvic-fin base. Pelvic-fin rays 8(57) total rays, surpassing anal-fin origin. Pelvic-fin rays with a sexually dimorphic pattern, which are absent in females, described below. Caudal-fin forked, upper and lower lobes similar in size. Principal caudal-fin rays 11+10(50) or 10+9(7); dorsal procurent rays 8(2), 9(8) or 11(2) and ventral procurent rays 7(4) or 8(8).

Branchiostegal rays 4(12). First gill arch with 1(11), 2(1) hypobranchial, 11(1), 12(10), or 13(1) ceratobranchial, 1(12) on cartilage between ceratobranchial and epibranchial, and 5(1) or 6(11) epibranchial gill-rakers. Supraneurals 3(2), 4(9), or 5(1). Total vertebrae 28(2) or 29(10).

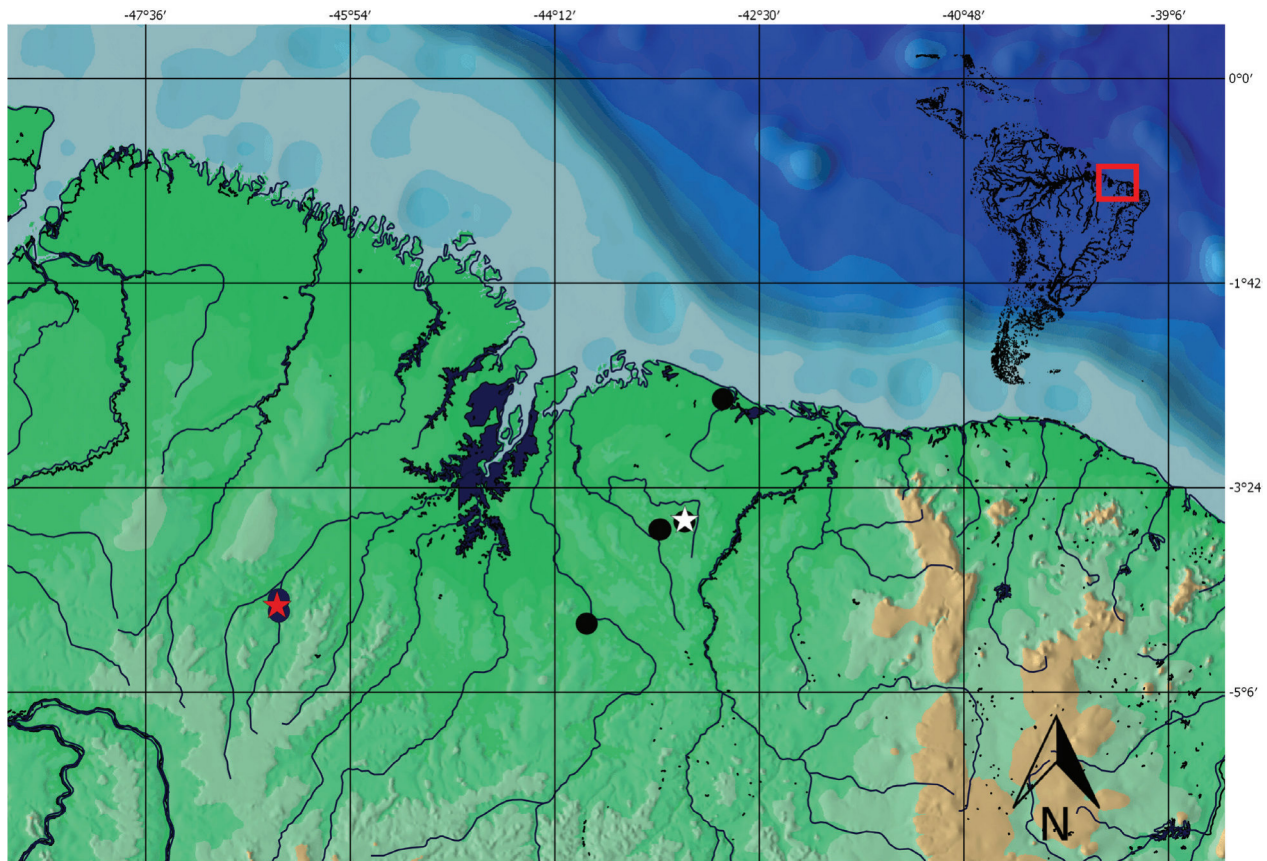


Figure 4. Geographical distribution of *Hyphessobrycon caru* sp. nov. and *H. piorskii*. Red star denotes Holotype and blue circles denote paratypes of *H. caru* sp. nov., and white star denotes Holotype and the black circles denote the known distribution of *H. piorskii*.

Colour in alcohol. Ground coloration light yellowish brown. Humeral region with few irregular inconspicuous vertically arranged chromatophores, sometimes very thin and inconspicuous humeral spot. Flank with chromatophores homogeneously scattered, more concentrated on posterior region to humeral spot, posterior region of dorsal-fin base origin and below mid-portion of trunk, between anal-fin origin and caudal peduncle. Ventral region lacking dark-brown chromatophores. Dark-brown chromatophores present on head and more concentrated on dorsal portion, becoming sparser on cheek and preopercular regions.

Dorsal-fin ground coloration hyaline, with conspicuous black or dark-brown spot located on anterior portion of fin, reaching about 6th ray, approximately between one-half to two-thirds of fin depth. Anal and caudal-fins hyaline. Caudal-fin with a darker, usually dark brown, posterior margin and on its base. Adipose-fin hyaline to light brown, with dark-brown or black chromatophores more concentrated on its dorsal portion, depending on the specimen preservation state. Pectoral and pelvic-fins hyaline; pelvic-fin with variable amounts of dark-brown pigmentation remaining depending on the specimen preservation state.

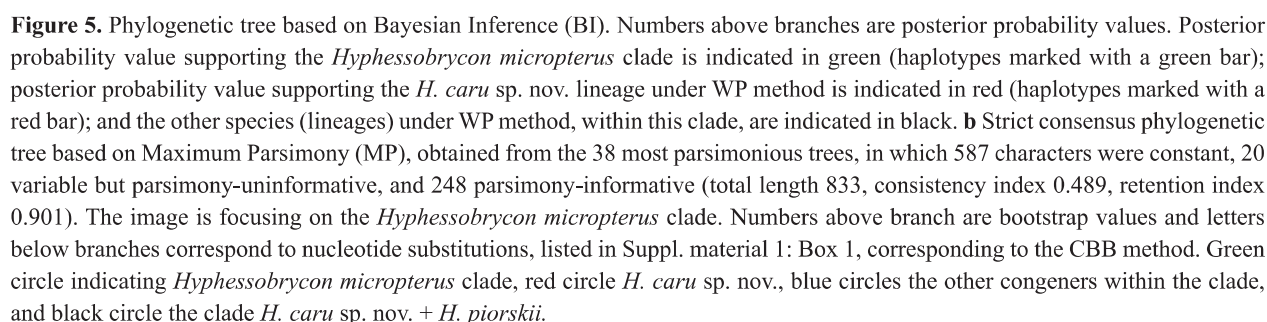
Sexual dimorphism. Mature males with small bone hooks on anal and pelvic-fin rays. Bone hooks absent on females. Anal-fin presenting bone hooks from 3rd, 4th, or

5th rays to the last ray. Number of hooks variable, increasing from the first to the last rays. Pelvic-fin presenting 2nd, 3rd, 4th, or 5th rays with 5, 6, or 7 smaller hooks.

Etymology. The specific epithet honors the term “Caru”. Caru is the name of an area (about 70.000 ha) inhabited by Brazilian native tribes from the ethnicities Guajá and Guajajara. People from this area use the Tupi language and have suffered consequences of European colonization and are under threat due to the pressure for exploration of the protected territory.

Geographic distribution. *Hyphessobrycon caru* sp. nov. has a restricted geographic distribution, being known only from the upper Pindaré river drainage, Mearim river basin, in the state of Maranhão, northeastern Brazil (Fig. 4). This species was never collected in the lower portions of this river drainage during 8 years of field trips conducted by EG and PB, including about 15 expeditions.

Molecular diagnosis (CBB). *Hyphessobrycon caru* sp. nov. belongs to the *H. micropterus* clade possessing 20 synapomorphic nucleotide substitutions: COI 73 (C→T), COI 88 (T→C), COI 217 (C→T), COI 274 (C→T), COI 298 (C→T), COI 334 (C→G), COI 338 (T→C), COI 370 (A→G), COI 418 (A→G), COI 433 (C→T), COI 439 (C→A), COI



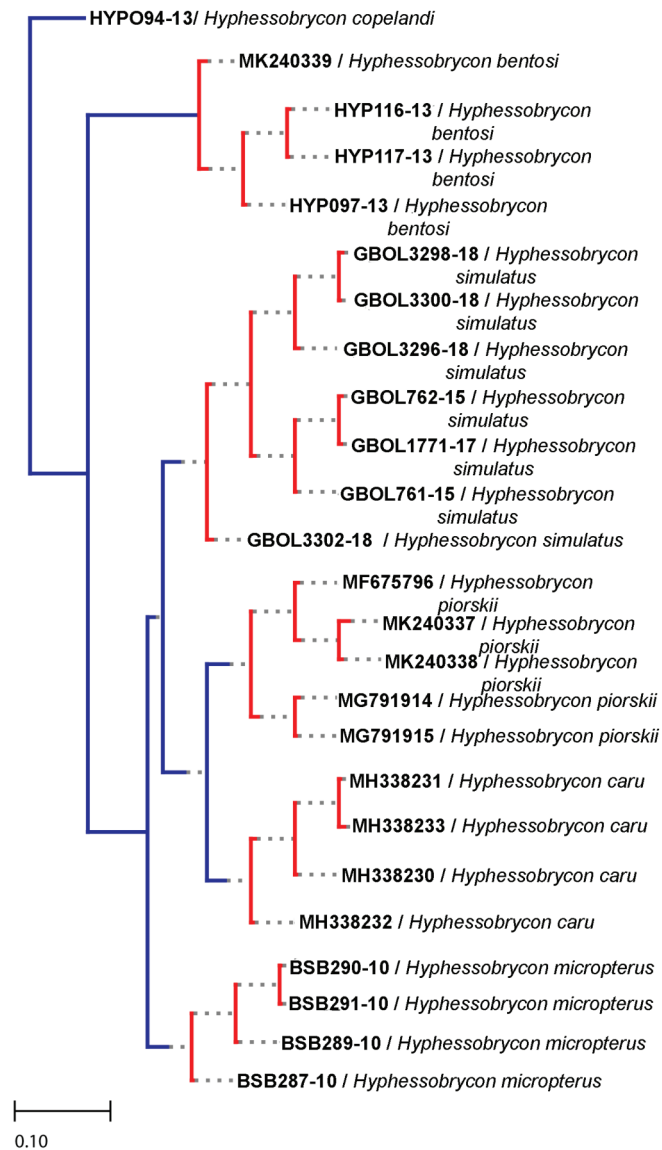


Figure 6. Species delimitation tree generated by the Bayesian Poisson Tree Processes (bPTP) model, using a fragment of the mitochondrial gene COI. The blue lines indicate branching processes among species, while red lines indicate branching processes within species.

457 (A→G), COI 469 (T→C), COI 478 (A→T), COI 559 (A→G), COI 562 (T→A), COI 592(A→G), COI 631 (A→T), COI 655 (A→C), COI 673 (A→C). It shares nine synapomorphic nucleotide substitutions with *H. piorskii*, which separate them from *H. simulatus* and *H. micropterus*: COI 181 (A→C), COI 208 (A→G), COI 245 (C→T), COI 325 (T→C), COI 349 (T→C), COI 436 (A→T), COI 472 (A→G), COI 538 (C→T), COI 556 (T→C). In addition, it has six unique nucleotide substitutions within the *H. micropterus* clade: COI 148 (C→T), COI 154 (C→T), COI 175 (T→C), COI 364 (G→A), COI 487 (T→C), COI 517 (A→G) (Fig. 5; Suppl. material 1: Box 1).

DBC. COI sequences support the existence of a new species of *Hyphessobrycon* inhabiting the Pindaré river basin in Maranhão state. After trimming, the final alignment yielded 680 base pairs with 159 polymorphic sites and 26

haplotypes. Average genetic distances were 18.3%, with the highest values between *H. epicharis* and *H. erythrostigma* (23.4%), while the lowest value (0.7%) was between *H. pyrrhonotus* and *H. erythrostigma* (Table 3). *Hyphessobrycon caru* sp. nov. is divergent on average 17.0% from the other taxa, with a minimum distance of 3.6% to *H. piorskii* and a maximum of 21.8% to *Pristella maxillaris* (Table 3).

WP and CBB. Both phylogenetic analysis based on BI and MP supported a clade comprising *H. caru* sp. nov., *H. micropterus*, *H. piorskii*, and *H. simulatus*, hereafter termed *Hyphessobrycon micropterus* clade, with maximum posterior probability value and 99% bootstrap value in BI and MP, respectively. *Hyphessobrycon caru* sp. nov. formed a single exclusive lineage with maximum posterior probability value (posterior probability = 1) and 99% bootstrap value in BI and MP, respectively.

Table 3. Kimura-2 parameters pairwise genetic distances among species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 <i>H. erythrostigma</i>																
2 <i>H. pyrrhonotus</i>	0.007															
3 <i>H. socolofi</i>	0.037	0.035														
4 <i>H. simulatus</i>	0.192	0.194	0.179													
5 <i>H. micropterus</i>	0.205	0.202	0.198	0.041												
6 <i>H. piorskii</i>	0.188	0.185	0.178	0.063	0.064											
7 <i>H. caru</i>	0.206	0.210	0.203	0.062	0.057	0.036										
8 <i>H. eques</i>	0.175	0.178	0.163	0.154	0.157	0.160	0.160									
9 <i>H. copelandi</i>	0.192	0.189	0.186	0.170	0.176	0.158	0.168	0.102								
10 <i>H. epicharis</i>	0.234	0.230	0.220	0.170	0.175	0.189	0.190	0.187	0.197							
11 <i>H. bentosi</i>	0.106	0.103	0.112	0.197	0.194	0.205	0.204	0.195	0.220	0.219						
12 <i>H. cf. sweglesi</i>	0.210	0.207	0.197	0.182	0.187	0.201	0.209	0.181	0.197	0.030	0.222					
13 <i>P. maxillaris</i>	0.225	0.232	0.206	0.201	0.211	0.220	0.218	0.194	0.213	0.180	0.202	0.183				
14 <i>M. hemigrammoides</i>	0.219	0.219	0.209	0.179	0.185	0.178	0.179	0.203	0.211	0.196	0.221	0.199	0.166			
15 <i>H. compressus</i>	0.214	0.218	0.215	0.202	0.208	0.198	0.208	0.198	0.201	0.212	0.212	0.212	0.203	0.215		
16 <i>H. panamensis</i>	0.210	0.213	0.209	0.202	0.199	0.179	0.187	0.215	0.229	0.221	0.213	0.218	0.204	0.208	0.145	
17 <i>H. flammeus</i>	0.201	0.201	0.198	0.169	0.174	0.186	0.204	0.203	0.205	0.192	0.200	0.189	0.206	0.200	0.170	0.196

These species delimitation analysis (WP and CBB) have identical results, delimitating four species within the *Hyphessobrycon micropterus* clade: *H. caru* sp. nov., *H. micropterus*, *H. piorskii*, and *H. simulatus* (Fig. 5a, b). The nucleotide substitutions supporting these four lineages within the *H. micropterus* clade, and the nucleotide substitutions supporting this clade are presented in Figure 5b and Suppl. material 1: Box 1. The combination of nucleotide substitutions diagnosing *H. caru* sp. nov. are presented in the molecular diagnosis section.

bPTP. This species delimitation analysis also indicates four lineages (species) within the *Hyphessobrycon micropterus* clade: *H. caru* sp.n., *H. micropterus*, *H. piorskii*, and *H. simulatus* (Fig. 6). This outcome was similar to the aforementioned results. The species included as outgroups (*H. bentosi* and *H. copelandi*) were also supported as independent lineages.

Discussion

Currently molecular techniques are frequently useful for solve species complexes and discover cryptic species (e.g. Bickford et al. 2006; Costa and Amorim 2011; Pereira et al. 2011; Adams et al. 2014; Costa-Silva 2015; Costa et al. 2012, 2014, 2017; Amorim 2018; Guimarães et al. 2018; Ottoni et al. 2019) and could be an excellent complement for traditional taxonomy (Kekkonen and Hebert 2014). DNA barcoding has demonstrated to be very efficient for delimiting species of *Hyphessobrycon*, mainly in groups with little morphological variation (i.e., cryptic species) (see Castro-Paz et al. 2014; Guimarães et al. 2018), preferably when applied together with other species delimitation methods, such as PAA, DBC, CBB, bPTP, and WP in an integrative taxonomy perspective (Guimarães et al. 2018). The recognition of different genetic patterns and lineages in groups with very similar morphology has been a common pattern in the tree of eukaryotic life. This is observed particularly often in species-rich genera, such

as in several Neotropical fishes (e.g. Pereira et al. 2011; Roxo et al. 2012; Castro-Paz et al. 2014; Melo et al. 2014, 2016a; Benzaquem et al. 2015; Benine et al. 2015; Ottoni et al. 2019). DNA techniques can help to uncover morphological hidden diversity (Bickford et al. 2006; Adams et al. 2014), delimiting a putative population or group of populations as an independent lineage (species), and, subsequently, through a more meticulous analysis of morphological features, morphological differences between cryptic species can be found.

The large number of the described *Hyphessobrycon* species (about 160 spp.), with new species described every year, reveal an astonishing diversity within the genus. During the past 10 years, about 50 new species have been described (Fricke et al. 2019). However, historically *Hyphessobrycon* species have been described only on the basis of morphological features, including differences in the pigmentation patterns and teeth numbers and morphology, using few individuals per species (e.g. Steindachner 1882; Eigenmann 1915; Zarske 2008, 2014; Bragança et al. 2015). Recently, DNA barcoding in characoid fishes has been used to discriminate species, identify new ones, and reveal that it is not always possible to differentiate species based solely on their morphology (Ornelas-Garcia et al. 2008; Pereira et al. 2011; Castro-Paz et al. 2014; Melo et al. 2014, 2016a; Benine et al. 2015).

Our results suggest a cryptic speciation in the rosy tetra clade, more specifically in a new clade here defined, the *Hyphessobrycon micropterus* clade, including *H. caru* sp. nov., *H. micropterus*, *H. piorskii*, and *H. simulatus*, so far only known from the Pindaré, Itapecuru, Munim, Preguiças, and São Francisco river drainages of Brazil and the coastal river basins of French Guiana and Suriname (Guimarães et al. 2018; Brito et al. 2019; Fricke et al. 2019; this study). The clade proposed here is supported by high node support values (maximum posterior probability value and 99% of bootstrap value in BI and MP, respectively). In addition, this clade was corroborated by 20 synapomorphic nucleotide substitutions (Fig. 5; Suppl. material 1: Box 1).

Hyphessobrycon caru sp. nov. is herein described within the *Hyphessobrycon micropterus* clade based on five different and independent methods of species delimitation (PAA, DBC, WP, CBB and bPTP), characterized by different criteria and assumptions. *Hyphessobrycon caru* sp. nov. is distinguished from all its congeners by a combination of unambiguous morphological character states [see Diagnosis (PAA)]. In our Bayesian phylogenetic analysis (Fig. 5A), haplotypes of *H. caru* sp. nov. formed a single exclusive clade with maximum posterior probability value (posterior probability = 1) (WP). Furthermore, the COI average genetic distance of *H. caru* sp. nov. when compared with the other taxa herein analyzed was 19.6% and its minimum COI genetic distance was 3.6% to *H. piorskii* (DBC). Considering this value, the threshold of *H. caru* sp. nov. would be greater than that inferred by delimitations among Neotropical fish species (2% according to Pereira et al. 2011). Moreover, *H. caru* sp. nov. was also molecularly diagnosed by six synapomorphic nucleotide substitutions (Fig. 5b; Suppl. material 1: Box 1), as well as, by a combination of other nucleotide substitutions (see CBB - molecular diagnosis), and corroborated by a bPTP analysis. Thus, it makes the hypothesis of this new species stronger from an integrative taxonomy perspective (see Dayrat 2005; de Queiroz 2007; Goldstein and Desalle 2010; Padial et al. 2010). Therefore, we recommend the use of integrative taxonomy for future taxonomic revisions and species descriptions when dealing with species complexes and groups containing possible cryptic species.

Comparative material

***Hyphessobrycon amandae*:** UFRJ 1557, 5 spcms, Jussara municipality, Goiás state, Brazil. ***H. bentosi*:** MCZ 20842, 1 spcm (Syntype), Óbidos municipality, Pará state, Brazil. ***H. bifasciatus*:** UFRJ 0068, 6 spcms, Maratáizes and Guarapari municipality, Espírito Santo state, Brazil. ***H. compressus*:** BMNH 1905.12.6.4-5, 2 spcms (Paratypes), Oaxaca state, México. ***H. copelandi*:** CAS 42683, 1 spcm (Syntype); MCZ 20771, 1 spcm (Syntype), Tabatinga municipality, Amazonas state, Brazil. ***H. eques*:** CICCAA 00715, 4 spcms (C&S); CICCAA 00710, 51 spcms, Tombos municipality Carangola river, Minas Gerais state, Brazil. ***H. erythrostigma*:** ANSP 70208, 1 spcm (Holotype), Peru and Brazil. ***H. epicharis*:** FMNH100609, 1 spcm (Paratype), Baria river, Amazonas, Venezuela. ***H. haraldschultzi*:** CICCAA 00873, 20 spcms, Ilha do Bananal municipality, Javaés river, Tocantins state, Brazil. ***H. hasemani*:** ANSP 39230, 1 spcm (Holotype), Guajaramirim municipality, Madeira river, Rondônia state, Brazil. ***H. micropterus*:** FMNH 57916, 1 spcm (Holotype), São Francisco river at Lagoa de Porto, Minas Gerais state, Brazil. ***H. piorskii*:** CICCAA 00695, 1 spcm (Holotype); CICCAA 00430, 15 spcms (Paratype); CICCAA 00431, 21 spcms (Para-

type); CICCAA 00696, 15 spcms (Paratype); CICCAA 00697, 16 spcms (C&S) (Paratype); CICCAA 00698, 6 spcms, 1 spcm (C&S) (Paratype); CICCAA 00750, 9 spcms (Paratype); CICCAA01654, 1 spcm (Paratype); CPUFMA 171664, 15 spcms (Paratype); UFRJ 11553, 6 spcms (Paratype), stream at the Anapurus municipality, Munim river, Maranhão state, Brazil. CICCAA 00089, 1 spcm (C&S) (Paratype); CICCAA 00881, 1 spcm (Paratype); CICCAA 01563, 1 spcm (Paratype); stream at Mata de Itamacaoca, Chapadinha municipality, Munim river, Maranhão state, Brazil. CICCAA 01382, 5 spcms (Paratype); CICCAA 02008, 12 (C&S) spcms (Paratype), stream at Mata Fome, Barreirinhas municipality, Preguiças river, Maranhão state, Brazil. ***H. pyrrhonotus*:** MZUSP 45714, 1 spcm (Holotype), Ereré river, Brazil. ***H. rosaceus*:** FMNH 52791, 1 spcm (Holotype), Gluck Island, Essequibo River, Guyana. ***H. werneri*:** MZUSP 42365, 1 spcm (Holotype), Santa Maria do Pará and São Miguel de Guamá municipality, Guamá river, Pará state, Brazil. CICCAA 00751, 1 spcm, Paragominas municipality, Candiru river, Pará state, Brazil. ***H. socolofi*:** MZUSP 13181, 1 spcm (Holotype), Barcelos municipality, Negro river, Amazonas state, Brazil.

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Supplementary material 1

Box 1

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Data type: DOCX file

Explanation note: List of nucleotide substitutions (synapomorphies and autapomorphies) from each lineage (species) and some crucial points of the cladogram of the Fig. 5B.

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