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

Erick Cristofore Guimarães[1,3,4], Pâmella Silva de Brito[1,3,4], Leonardo Manir Feitosa[2], Luis Fernando Carvalho Costa[3], Felipe Polivanov Ottoni[1,4,5,6]

1 *Universidade Federal do Maranhão, Programa de Pós-Graduação em Biodiversidade e Biotecnologia da Amazônia Legal. Av. dos Portugueses 1966, Cidade Universitária do Bacanga, CEP 65080-805, São Luís, MA, Brazil*

2 *Universidade Federal de Pernambuco, Programa de Pós-Graduação em Biologia Animal. Av. Professor Moraes Rego 1235, Cidade Universitária, CEP 50670-901, Recife, PE, Brazil*

3 *Universidade Federal do Maranhão, Departamento de Biologia, Laboratório de Genética e Biologia Molecular, Av. dos Portugueses 1966, Cidade Universitária do Bacanga, CEP 65080-805, São Luís, MA, Brazil*

4 *Universidade Federal do Maranhão, Laboratório de Sistemática e Ecologia de Organismos Aquáticos, Centro de Ciências Agrárias e Ambientais, Campus Universitário, CCAA, BR-222, KM 04, S/N, Boa Vista, CEP 65500-000, Chapadinha, MA, Brazil*

5 *Universidade Federal do Maranhão, Programa de Pós-Graduação em Biodiversidade e Conservação. Av. dos Portugueses 1966, Cidade Universitária do Bacanga, CEP 65080-805, São Luís, MA, Brazil*

6 *Universidade Federal do Maranhão, Programa de Pós-graduação em Ciências Ambientais, Centro de Ciências Agrárias e Ambientais, Campus Universitário, CCAA, BR-222, KM 04, S/N, Boa Vista, CEP 65500-000, Chapadinha, MA, Brazil*

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Corresponding author: *Erick Cristofore Guimarães* (erick.ictio@yahoo.com.br)

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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).

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	*Hyphessobrycon erythrostigma*	INPA 37681·HERY1	Tabatinga/Amazonas/Brazil	HYP076·13
2	*Hyphessobrycon erythrostigma*	INPA 37681·HERY10	Tabatinga/Amazonas/Brazil	HYP077·13
3	*Hyphessobrycon erythrostigma*	INPA 37681·HERY2	Tabatinga/Amazonas/Brazil	HYP078·13
4	*Hyphessobrycon erythrostigma*	INPA 37681·HERY3	Tabatinga/Amazonas/Brazil	HYP079·13
5	*Hyphessobrycon pyrrhonotus*	INPA 37672·TRO10	Santa Isabel do Rio Negro/Amazonas/Brazil	HYP040·13
6	*Hyphessobrycon pyrrhonotus*	INPA 37672·TRO11	Santa Isabel do Rio Negro/Amazonas/Brazil	HYP041·13
7	*Hyphessobrycon pyrrhonotus*	–	Barcelos/Amazonas/Brazil	HYP157·13
8	*Hyphessobrycon pyrrhonotus*	–	Barcelos/Amazonas/Brazil	HYP158·13
9	*Hyphessobrycon socolofi*	INPA_39530·6152	Barcelos/Amazonas/Brazil	HYP131·13
10	*Hyphessobrycon socolofi*	INPA_39530·6155	Barcelos/Amazonas/Brazil	HYP134·13
11	*Hyphessobrycon socolofi*	INPA_39530·6178	Barcelos/Amazonas/Brazil	HYP135·13
12	*Hyphessobrycon socolofi*	INPA 39530·BCR8	Barcelos/Amazonas/Brazil	HYP148·13
13	*Hyphessobrycon copelandi*	INPA_37683·TU1	Tabatinga/Amazonas/Brazil	HYP094·13
14	*Hyphessobrycon copelandi*	INPA_37683·TU2	Tabatinga/Amazonas/Brazil	HYP095·13
15	*Hyphessobrycon copelandi*	INPA_37683·TU3	Tabatinga/Amazonas/Brazil	HYP096·13
16	*Hyphessobrycon eques*	INPA_37678·IC2	Santarém/Pará/Brazil	HYP070·13
17	*Hyphessobrycon eques*	INPA_37679·PE1	Macapá/Amapá/Brazil	HYP071·13
18	*Hyphessobrycon eques*	INPA_37680·AL1	Parintins/Amazonas/Brazil	HYP072·13
19	*Hyphessobrycon eques*	OL·0544	Bonito/Mato Grosso do Sul/Brazil	DSMIS077·09
20	*Hyphessobrycon epicharis*	INPA_37665·JUF1	São Gabriel da Cachoeira/Amazonas/Brazil	HYP002·13
21	*Hyphessobrycon epicharis*	INPA_37665·JUF3	São Gabriel da Cachoeira/Amazonas/Brazil	HYP004·13
22	*Hyphessobrycon epicharis*	INPA_37665·JUF4	São Gabriel da Cachoeira/Amazonas/Brazil	HYP005·13
23	*Hyphessobrycon epicharis*	INPA_37665·JUF8	São Gabriel da Cachoeira/Amazonas/Brazil	HYP006·13
24	*Hyphessobrycon compressus*	CINV·NEC7411	Flores Magon/Campeche/México	FYPM054·10
25	*Hyphessobrycon compressus*	ECOCH	Hatie ville/Belize/Belize	MXV765·15
26	*Hyphessobrycon compressus*	ECOCH	Hatie ville/Belize/Belize	MXV766·15
27	*Hyphessobrycon compressus*	ECOCH	Hatie ville/Belize/Belize	MXV767·15
28	*Hyphessobrycon bentosi*	INPA_37684·5939	Barcelos/Amazonas/Brazil	HYP097·13
29	*Hyphessobrycon bentosi*	INPA_37684·5940	Barcelos/Amazonas/Brazil	HYP098·13
30	*Hyphessobrycon bentosi*	INPA_39527·BA1	–	HYP116·13
31	*Hyphessobrycon bentosi*	INPA_39527·BA2	–	HYP117·13
32	*Hyphessobrycon bentosi*	CICCAA02349	Santarém/Pará/Brazil	**MK240339**
33	*Hyphessobrycon bentosi*	CICCAA02350	Santarém/Pará/Brazil	**MK240340**
34	*Hyphessobrycon bentosi*	CICCAA02351	Santarém/Pará/Brazil	**MK240341**
35	*Hyphessobrycon simulatus*	MHNG 2743.087	Pisiemoengo/Commewijne/Suriname	**GBOL761-15**
36	*Hyphessobrycon simulatus*	MHNG 2743.087	Pisiemoengo/Commewijne/Suriname	**GBOL762-15**
37	*Hyphessobrycon simulatus*	–	Sinnamary/Cayenne/French Guiana	**GBOL1771-17**
38	*Hyphessobrycon simulatus*	MHNG 2735.007	Sinnamary/Cayenne/French Guiana	**GBOL3296-18**
39	*Hyphessobrycon simulatus*	MHNG 2757.080	Kourou/Cayenne/French Guiana	**GBOL3298-18**
40	*Hyphessobrycon simulatus*	MHNG 2759.026	Kaw/Cayenne/French Guiana	**GBOL3300-18**
41	*Hyphessobrycon simulatus*	MHNG 2759.026	Kaw/Cayenne/French Guiana	**GBOL3301-18**
42	*Hyphessobrycon simulatus*	MHNG 2759.035	Régina/ Cayenne/French Guiana	**GBOL3302-18**
43	*Hyphessobrycon* cf. *sweglesi*	INPA_37668·JAR3	São Gabriel da Cachoeira/Amazonas/Brazil	HYP026·13
44	*Hyphessobrycon* cf. *sweglesi*	INPA_37668·JAR4	São Gabriel da Cachoeira/Amazonas/Brazil	HYP027·13
45	*Hyphessobrycon* cf. *sweglesi*	INPA_37668·JAR5	São Gabriel da Cachoeira/Amazonas/Brazil	HYP028·13
46	*Hyphessobrycon* cf. *sweglesi*	INPA_37668·JAR7	São Gabriel da Cachoeira/Amazonas/Brazil	HYP030·13
47	*Hyphessobrycon micropterus*	–	Várzea da Palma/Minas Gerais/Brazil	BSB287·10
48	*Hyphessobrycon micropterus*	–	Várzea da Palma/Minas Gerais/Brazil	BSB288·10
49	*Hyphessobrycon micropterus*	–	Várzea da Palma/Minas Gerais/Brazil	BSB289·10
50	*Hyphessobrycon micropterus*	–	Várzea da Palma/Minas Gerais/Brazil	BSB290·10
51	*Hyphessobrycon piorskii*	CICCAA00725·1	Chapadinha/Maranhão/Brazil	MF765796
52	*Hyphessobrycon piorskii*	CICCAA00726·1	Chapadinha/Maranhão/Brazil	MF765797
53	*Hyphessobrycon piorskii*	CICCAA01650·1	Barreirinhas/Maranhão/Brazil	MG791915
54	*Hyphessobrycon piorskii*	CICCAA01651·1	Barreirinhas/Maranhão/Brazil	MG791914
55	*Hyphessobrycon piorskii*	CICCAA02164·1	Codó/Maranhão/Brazil	**MK240337**
56	*Hyphessobrycon piorskii*	CICCAA02164·4	Codó/Maranhão/Brazil	**MK240338**
57	*Hyphessobrycon caru*	CICCAA00748·1	Buriticupu/Maranhão/Brazil	**MH338230**
58	*Hyphessobrycon caru*	CICCAA00749·1	Buriticupu/Maranhão/Brazil	**MH338231**
59	*Hyphessobrycon caru*	CICCAA02300·1	Buriticupu/Maranhão/Brazil	**MH338232**
60	*Hyphessobrycon caru*	CICCAA02301·1	Buriticupu/Maranhão/Brazil	**MH338233**
61	*Pristella maxillaris*	–	–	KU568982.1
62	*Pristella maxillaris*	–	–	KU568981.1
63	*Pristella maxillaris*	–	Marlborough/Pomeroon·Supenaam/Guyana	TZGAA025·06
64	*Pristella maxillaris*	–	Santa Cruz/Barima·Waini/Guyana	TZGAA178·06
65	*Moenkhausia hemigrammoides*	INPA38532·PR1	Guyana	HYP101·13
66	*Moenkhausia hemigrammoides*	INPA_38532·PR2	Guyana	HYP102·13
67	*Moenkhausia hemigrammoides*	INPA_38532·PR3	Guyana	HYP103·13
68	*Hyphessobrycon panamensis*	STRI·05303	Cocle/Panama	BSFFA760·07
69	*Hyphessobrycon flammeus*	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, procurrent 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 15µl containing 1× Polymerase buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.2 uM of each primer, 1U of Taq Polymerase (Invitrogen), 100 ηg 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 (Avise 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. werneri* 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 procurrent 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.

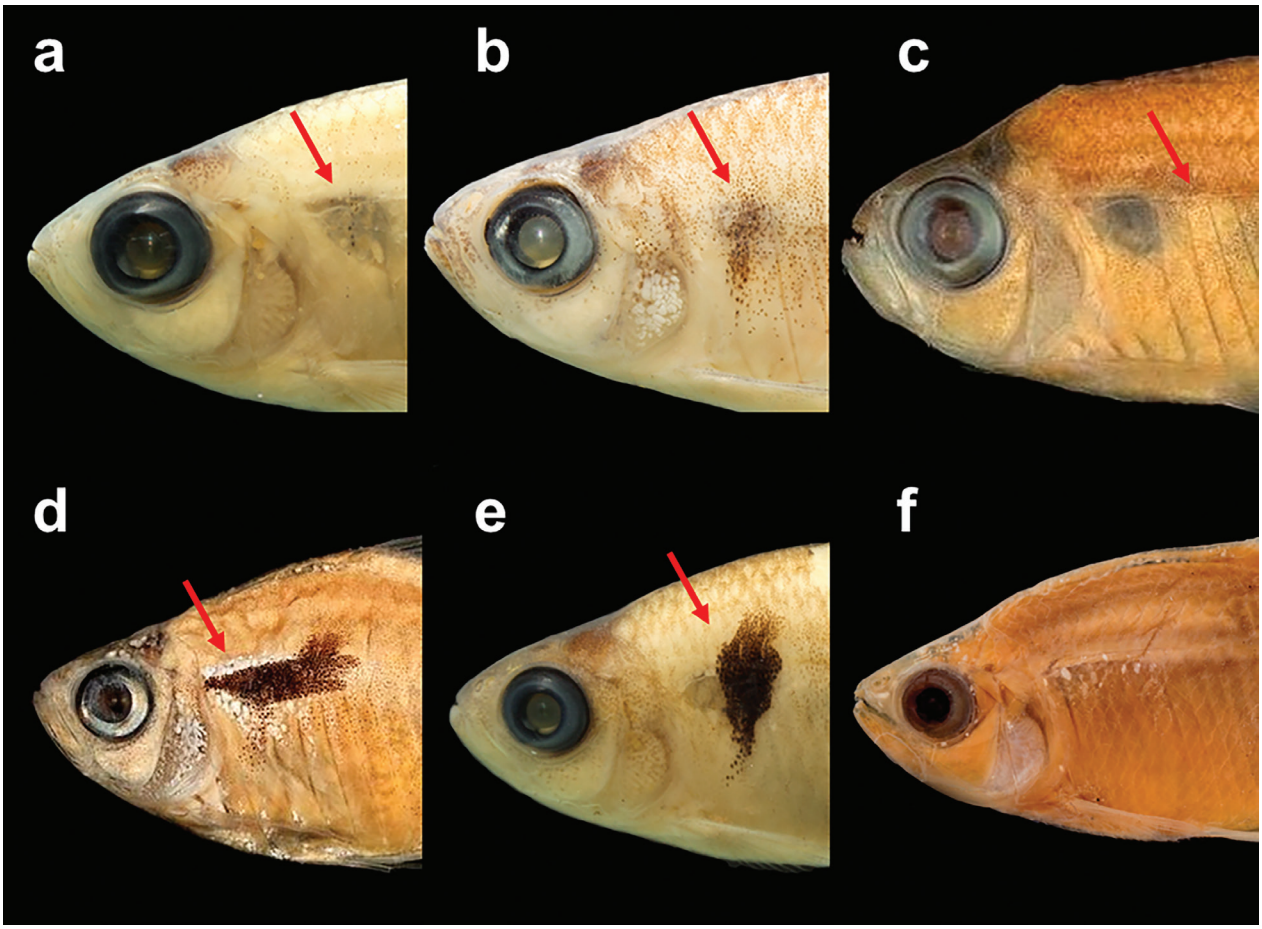

Figure 2. Humeral spot of: **a** *Hyphessobrycon caru*, holotype, CICCAA 02286 **b** *H. piorskii*, holotype, CICCAA 00695 **c** *H. pyrrhonotus*, holotype, MZUSP 45714 **d** *H. werneri*, holotype, MZUSP 42365 **e** *H. eques*, CICCAA 00300 **f** *H. compressus*, paratype, MHNG 2181.076.

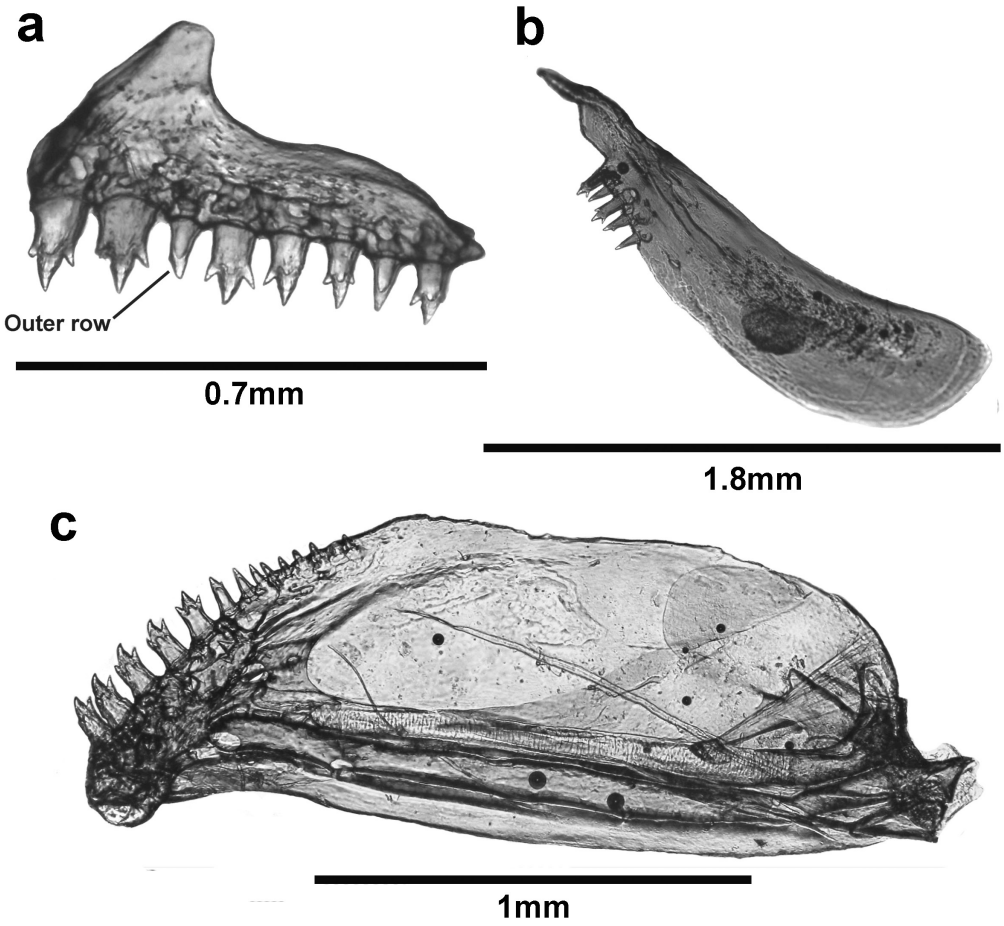

Figure 3. *Hyphessobrycon caru* sp. nov., jaw suspensory, CICCAA 00697, paratype, 19.3 mm SL: premaxillary (**a**), maxillary (**b**), and dentary (**c**).

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 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).

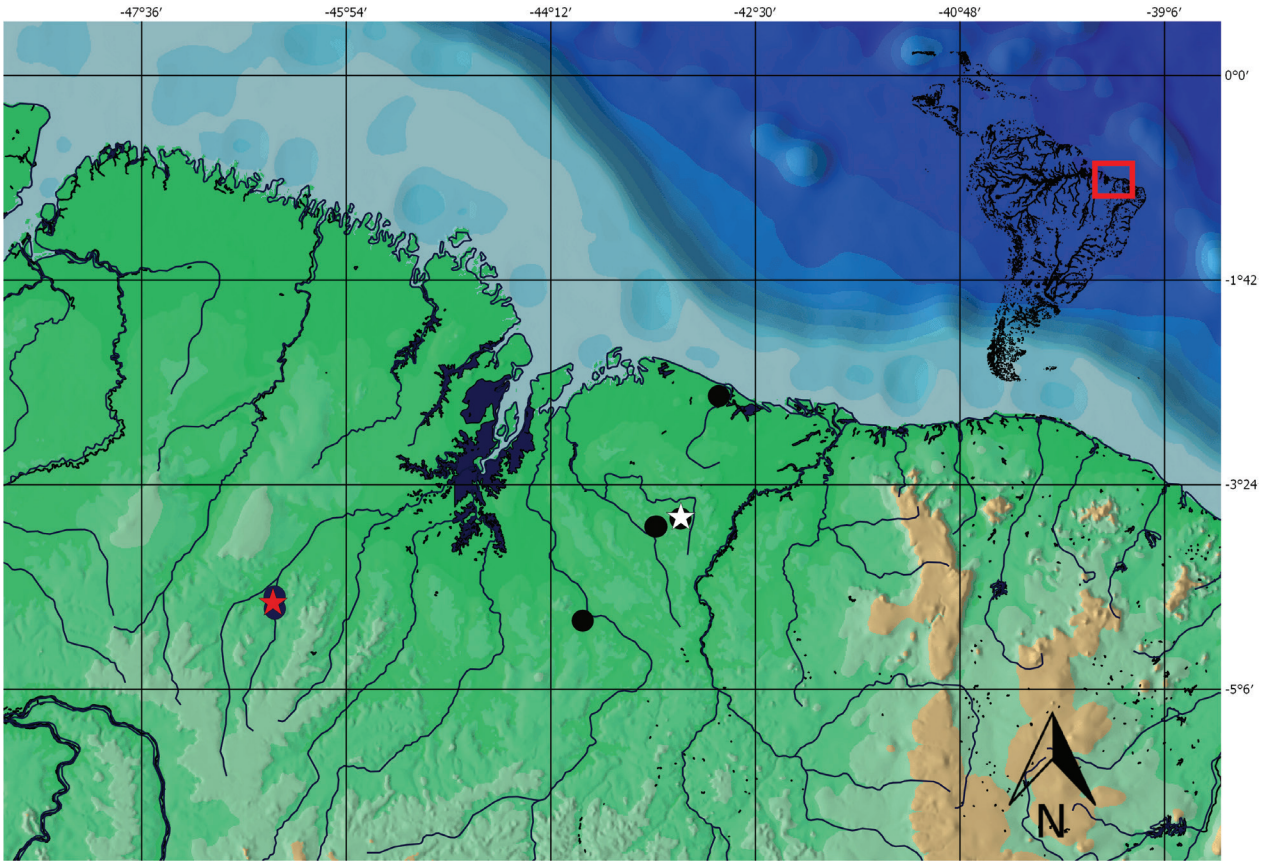

Figure 4. Geographical distribution of *Hyphessobrycon caru* sp. nov. and *H. piorskii*. Red star denotes Holotype and blue circles denote paratypes of *H. caru* sp. nov., and white star denotes Holotype and the black circles denote the known distribution of *H. piorskii*.

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

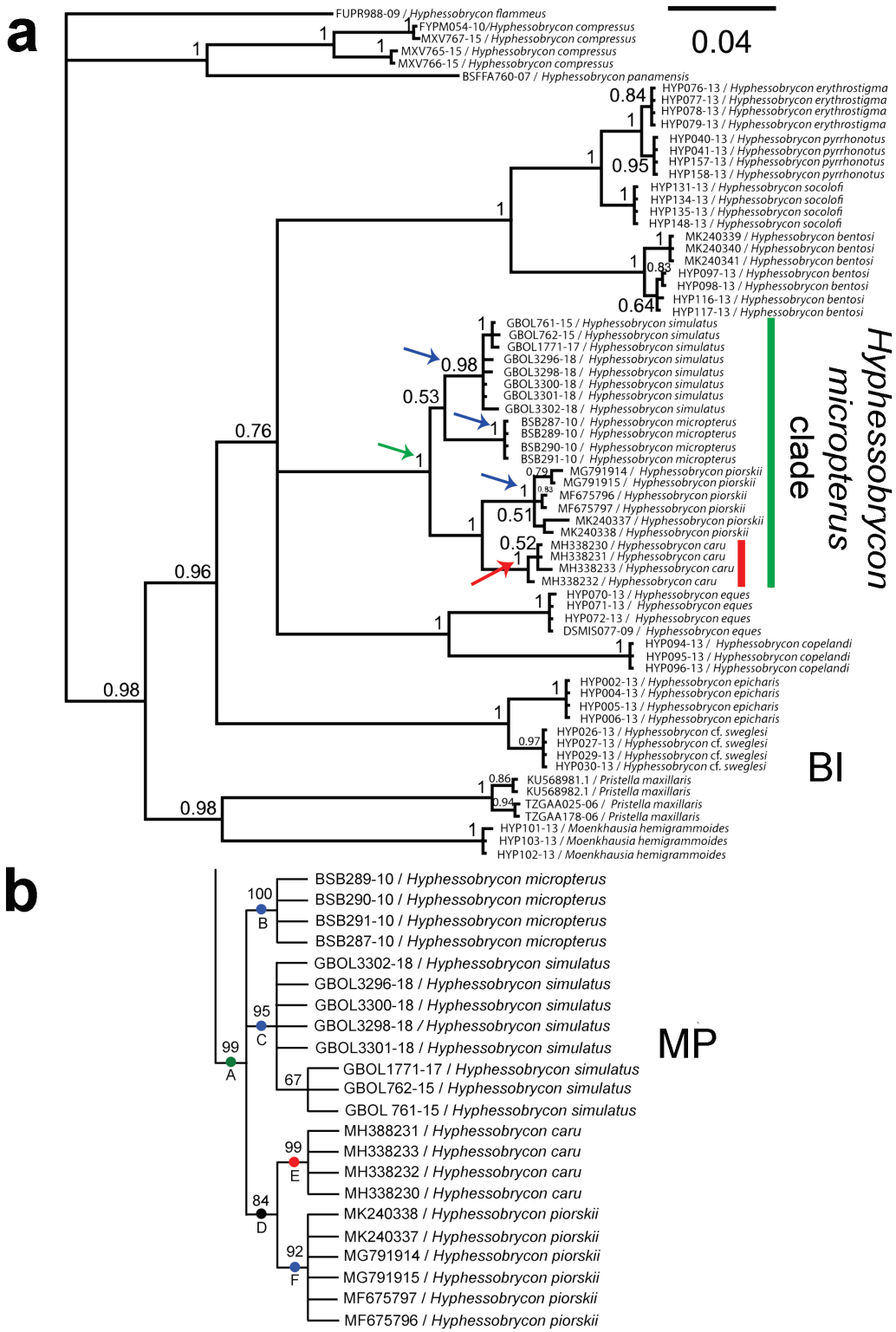

Figure 5. Phylogenetic tree based on Bayesian Inference (BI). Numbers above branches are posterior probability values. Posterior probability value supporting the *Hyphessobrycon micropterus* clade is indicated in green (haplotypes marked with a green bar); posterior probability value supporting the *H. caru* sp. nov. 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 values and letters below branches correspond to nucleotide substitutions, listed in Suppl. material 1: Box 1, corresponding to the CBB method. Green circle indicating *Hyphessobrycon micropterus* clade, red circle *H. caru* sp. nov., blue circles the other congeners within the clade, and black circle the clade *H. caru* sp. nov. + *H. piorskii.*

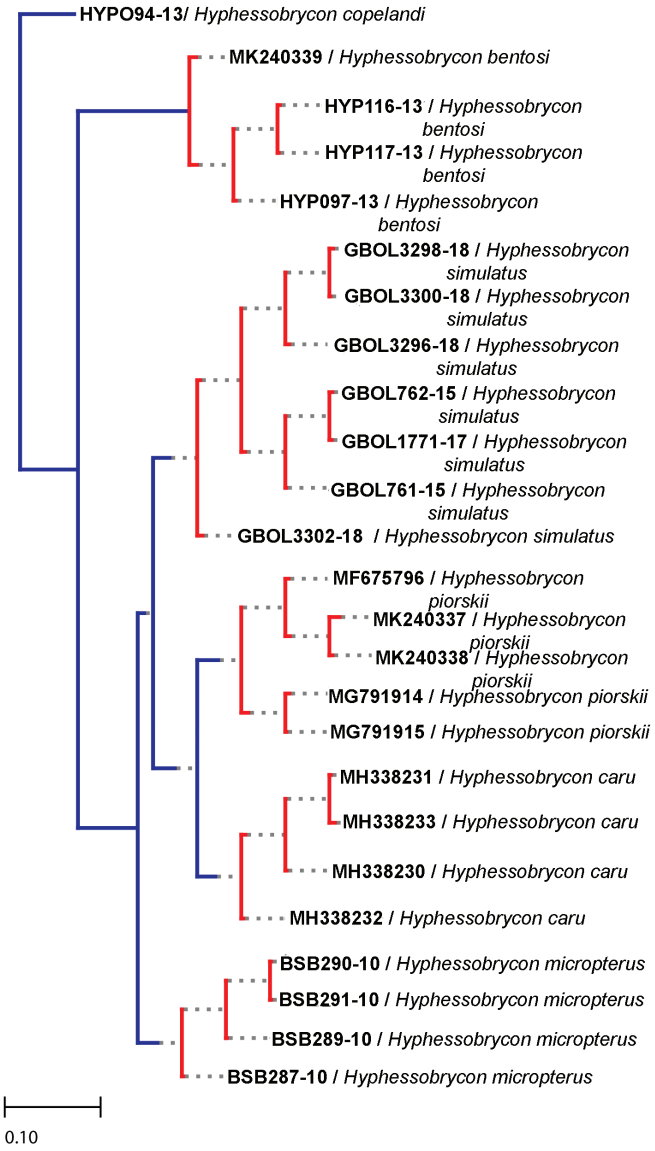

Figure 6. Species delimitation tree generated by the Bayesian Poisson Tree Processes (bPTP) model, using a fragment of the mitochondrial gene COI. The blue lines indicate branching processes among species, while red lines indicate branching processes within species.

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	*H. erythrostigma*																
2	*H. pyrrhonotus*	0.007															
3	*H. socolofi*	0.037	0.035														
4	*H. simulatus*	0.192	0.194	0.179													
5	*H. micropterus*	0.205	0.202	0.198	0.041												
6	*H. piorskii*	0.188	0.185	0.178	0.063	0.064											
7	***H. caru***	0.206	0.210	0.203	0.062	0.057	0.036										
8	*H. eques*	0.175	0.178	0.163	0.154	0.157	0.160	0.160									
9	*H. copelandi*	0.192	0.189	0.186	0.170	0.176	0.158	0.168	0.102								
10	*H. epicharis*	0.234	0.230	0.220	0.170	0.175	0.189	0.190	0.187	0.197							
11	*H. bentosi*	0.106	0.103	0.112	0.197	0.194	0.205	0.204	0.195	0.220	0.219						
12	*H.* cf. *sweglesi*	0.210	0.207	0.197	0.182	0.187	0.201	0.209	0.181	0.197	0.030	0.222					
13	*P. maxillaris*	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	*M. hemigrammoides*	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	*H. compressus*	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	*H. panamensis*	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	*H. flammeus*	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 synaphomorphic 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 (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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Supplementary material 1

Box 1

Authors: Erick Cristofore Guimarães, Pâmella Silva de Brito, Leonardo Manir Feitosa, Luis Fernando Carvalho Costa, Felipe Polivanov Ottoni

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.

Link: https://doi.org/10.3897/zse.95.34069.suppl1