Molecular phylogenetics of the Neotropical fish family Prochilodontidae (Teleostei: Characiformes)

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ABSTRACT

Migratory detritivores of the characiform family Prochilodontidae occur throughout the freshwaters of much of South America. Prochilodontids often form massive populations and many species achieve substantial body sizes; a combination that makes them one of the most commercially important fish groups on the continent. Their economic significance notwithstanding, prochilodontids have never been the subject of a comprehensive molecular phylogenetic analysis. Using three mitochondrial and three nuclear loci spanning all prochilodontid species, we generated a novel phylogenetic hypothesis for the family. Our results strongly support monophyly of the family and the three included genera. A novel, highly supported placement of *Ichthyoelephas* sister to the clade containing *Prochilodus* and *Semaprochilodus* diverges from a previous morphological hypothesis. Most previously hypothesized interspecific relationships are corroborated and some longstanding polytomies within *Prochilodus* and *Semaprochilodus* are resolved. The morphologically similar *P. brevis*, *P. lacustris*, *P. nigricans* and *P. rubrotaeniatus* are embedded within what is herein designated as the *P. nigricans* group. Species limits and distributions of these species are problematic and the group clearly merits taxonomic revision.

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1. Introduction

The megadiverse ostariophysian order Characiformes includes over 2000 species in Africa, Central and South America ranging in size from miniature Neotropical tetras to the African tigerfish, which exceeds one meter in length. Recent molecular phylogenetic studies have focused on the inter- and intrafamilial relationships of several characiform lineages (Ortí et al., 2008; Oliveira et al., 2011; Tagliacollo et al., 2012; Abe et al., 2013; Arroyave et al., 2013; Mariguela et al., 2013; Melo et al., 2014, 2016; Thompson et al., 2014; Thomaz et al., 2015) which previously had received only morphological attention. In many cases, molecular and morphological phylogenetic analyses agree within Characiformes, as in the strong support returned by both datatypes for the monophyly of the superfamily Anostomoidea, which includes the families Anostomidae, Chilodontidae, Curimatidae, and Prochilodontidae (Vari, 1983; Sidlauskas, 2008; Oliveira et al., 2011; Dillman et al.,

2015). In other instances, molecular and morphological hypotheses diverge, as in the African family Alestidae (Zanata and Vari, 2005; Arroyave and Stiassny, 2011). Notwithstanding these advances, the position of many characiform families (e.g. Crenuchidae, Ctenoluciidae, Lebiasinidae) remains uncertain, as do their intergeneric relationships. These discordances and uncertainties demonstrate the need to reexamine all characiform families via robust molecular datasets and phylogenies in order to understand the evolution within this major teleost lineage.

The characiform superfamily Anostomoidea with four trophically diverse families and about 300 species has received extensive systematic and revisionary treatment (Vari, 1983; Sidlauskas, 2008; Dillman et al., 2015) and its monophyly seems certain. However, its supra- and intrafamilial relationships have been explored in detail solely with morphology (Vari, 1989; Vari et al., 1995; Castro and Vari, 2004; Sidlauskas and Vari, 2008) and merit molecular examination. A recent molecular analysis of Chilodontidae (Melo et al., 2014), for example, reinforced the hypothesis of the monophyly of the two genera, revealed several cryptic species, suggested potential synonymies, and slightly differed from the morphological hypothesis (Vari et al., 1995). That analysis further demonstrates the utility of molecular data in taxonomic and

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evolutionary studies within characiforms and indicates that comparable studies of the three other families in Anostomoidea will yield similar insights.

Herein, we generate the first comprehensive molecular phylogeny of the detritivorous family Prochilodontidae, known as *curimbatás* and *jaraquis* in Portuguese, *bocachicos* in Spanish, and flannel-mouth characiforms in English (Castro and Vari, 2003). The family includes three distinctive genera: the remarkable *Ichthyoelephas* characterized by a unique, highly developed upper lip (Fig. 1a) in rivers west of the Andes in Colombia and Ecuador plus two abundant, migratory, commercially important genera, *Prochilodus* (13 species, Fig. 1b–f) and *Semaprochilodus* (6 species, Fig. 1g–h). *Prochilodus* occurs in all major South American river systems on both sides of the Andes whereas *Semaprochilodus* is broadly distributed east of the Andes through the Amazon,

Tocantins and Orinoco basins and some coastal rivers draining the Guiana Shield.

Prochilodontidae is readily distinguished from all other fishes by the presence of numerous rows of relatively small teeth attached to the fleshy lips and structurally unique upper and lower jaws that support the expanded lips. These lips form an evertable (outwards extendable) oral disk (Castro and Vari, 2004) which, in combination with the multiple tooth rows, function to scrape periphyton and detritus off submerged surfaces and to suck detritus from the bottom of water bodies (Fig. 1c) (Bowen, 1983; Bowen et al., 1984). Prochilodontids feed towards the base of the food chain and form a major link in the carbon flow within aquatic systems across much of the Neotropics (Taylor et al., 2006). Their specialization on a hyperabundant energy resource, periphyton and detritus, make possible the often large body sizes and enormous
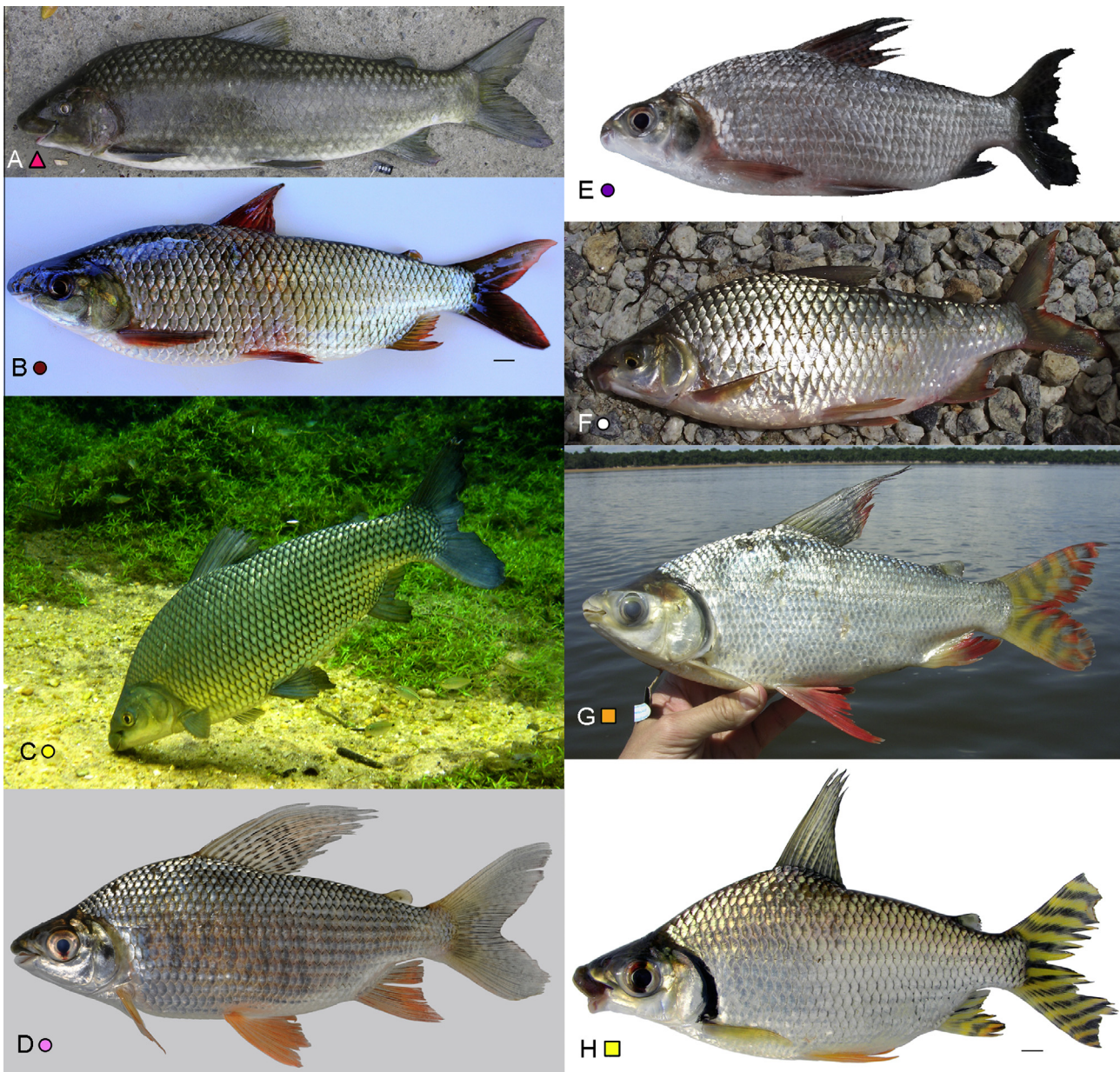

Fig. 1. Live and recently captured specimens of Prochilodontidae. (A) *Ichthyoelephas longirostris*, ANSP 192865, Colombia, Río Magdalena basin; (B) *Prochilodus harttii*, Brazil, Rio Jequitinhonha; (C) *P. lineatus*, Brazil, Rio Paraguay basin; (D) *P. nigricans*, Brazil, Rio Xingu, Amazon basin; (E) *P. rubrotaeniatus*, Brazil, Rio Branco, Amazon basin; (F) *P. vimboides*, Brazil, Rio Mucuri; (G) *Semaprochilodus laticeps* (right after death), MCNG 51963, Venezuela, Río Orinoco basin; (H) *S. varii*, ANSP 187435, Suriname, Marowijne River basin. Scale bar = one centimeter. Photos by M.H. Sabaj Pérez (A, D, G and H), T.C. Pessali (B), J. Sabino (C), B.F. Melo (E) and L.M. Sarmento-Soares (F).

populations (Carolsfeld et al., 2003) of many species in the family. Such populations, in turn, allow these species to sustain the most important commercial fisheries in many South American river systems (Bayley and Petrere, 1989; Ribeiro and Petrere, 1990; Camargo and Petrere, 2001; Garcia et al., 2009). Due to their large bodies and high abundance, prochilodontids rank among the most important artisanal food resources in many regions across South America, resulting in anthropogenically induced population declines in some basins (e.g. *Prochilodus lineatus* in the upper Rio Paraguay; Mateus et al., 2004). Prochilodontids additionally act as ecosystem engineers (Flecker, 1996), and their removal can significantly impact broader riverine aquatic faunas (Ribeiro and Petrere, 1990; Taylor et al., 2006).

Aside from their unusual feeding apparatus and incredible biomass, prochilodontids are famous for their large seasonal migrations which can span many hundreds of river kilometers (Paiva and Bastos, 1981; Godinho and Kynard, 2006). As a consequence, natural populations of some prochilodontid species span enormous geographic ranges. For example, a study of the population structure in *Prochilodus* (Sivasundar et al., 2001) suggested a remarkable intraspecific genetic homogeneity of resident prochilodontid species throughout each of the Magdalena, Orinoco, and Amazon basins, with the major watershed boundaries structuring this diversity. Recent initiatives to dam major tributaries of the Amazon basin (Winemiller et al., 2016) raised serious concerns that these barriers will diminish, if not eliminate, some flooded downstream areas and associated marginal lagoons considered crucial for the maintenance of genetic variability in *Prochilodus* (Melo et al., 2013). These major impoundments will also impede, and perhaps eliminate, the long distance migrations prevalent in these ecologically important fishes. Population fragmentation will likely have consequences for the reproduction and life history of one of the most important components of artisanal and commercial fisheries in South America.

Despite the economic importance of the Prochilodontidae, the systematics of this group remains incompletely resolved. Based on the examination of six species, Vari (1983) proposed the monophyly of the Prochilodontidae supported by 18 synapomorphies related to dentition and cranial osteology. In an extensive morphological-based phylogenetic study and taxonomic revision involving all prochilodontid species, Castro and Vari (2004) proposed 40 additional synapomorphies for the family, identified dozens of characters separating the three recognized genera, generated generic and interspecific phylogenies, and hypothesized *Prochilodus* as the sister to a clade containing *Ichthyoelephas* and *Semaprochilodus* (Fig. 2). Despite their broad osteological sampling, Castro and Vari (2004) were able to identify only a limited number of characters informative as to the relationships within *Prochilodus* and *Semaprochilodus* and, thus, only partially resolved the intrageneric phylogenies.

This lack of intrageneric resolution derives from the remarkable morphological similarity between the species within each genus, which are differentiated primarily in scale counts, body morphometrics, nuanced aspects of coloration, and most significantly the watersheds to which they are endemic. Morphological stasis among species in Prochilodontidae may stem from a primarily allopatric speciation mechanism as suggested by their drainage-wide distributions and their low levels of sympatry. Such allopatric separation of gene pools in combination with the ecological similarity of all prochilodontids would imply no drive for major morphological evolution during speciation. Although biologically predictable, this lack of informative morphological species-level variation presents a significant obstacle in reconstructing the evolutionary history within *Prochilodus* and *Semaprochilodus* based solely on such data. Molecular approaches, therefore, offer the surest path forward.
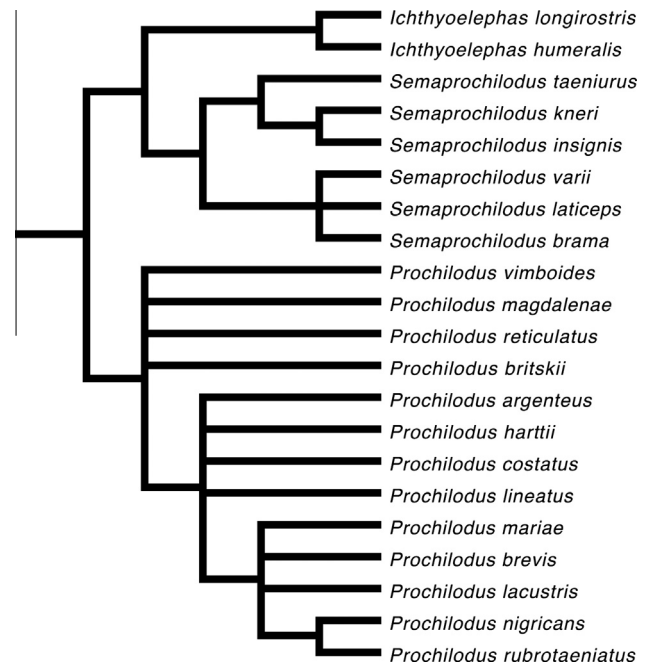

Fig. 2. Prochilodontid relationships hypothesized by Castro and Vari (2004).

In this study, we combine mitochondrial and nuclear loci to revisit the phylogenetics of the Prochilodontidae and advance our understanding of intrafamilial evolution. We develop the first phylogeny for the family using both Bayesian and likelihood methods. Using complete taxon sampling, we test Vari's (1983) hypothesis of monophyly of the family, evaluate Castro and Vari's (2004) hypotheses of relationships among the genera and species, and present the first fully resolved species-level phylogeny for the family.

2. Material and methods

2.1. Taxon sampling

We included 55 individuals representing all 21 species in the three genera of the Prochilodontidae and 22 related taxa from the other three anostomoid families (Anostomidae, Chilodontidae, Curimatidae), three families previously hypothesized to be closely related to Anostomoidea (Hemiodontidae, Parodontidae and Serrasalmidae), and *Brycon pesu*, which we used to root the trees. We used tissues preserved in 95% ethanol or a saturated DMSO/NaCl solution, primarily from specimens deposited in museum and university collections (Table 1; abbreviations follow Sabaj Pérez, 2014). Fig. 3 indicates sampling localities for each analyzed prochilodontid species and was produced via QGIS 2.2.0-Valmiera (www.qgis.org).

2.2. Multilocus sequencing

We extracted DNA from muscle tissue or fins with either a DNeasy Tissue kit (Qiagen Inc.) following manufacturer's instructions or a modified NaCl extraction protocol adapted from Lopera-Barrero et al. (2008). Following recent phylogenetic studies with characiforms (Oliveira et al., 2011; Abe et al., 2013; Melo et al., 2014), we amplified partial sequences of the mitochondrial genes *16S rRNA* (16S, 510 bp), *cytochrome oxidase C subunit 1* (COI, 658 bp) and *cytochrome B* (Cytb, 991 bp) using one round of polymerase chain reaction (PCR). Additionally, we obtained

Table 1
Voucher specimens and locality information of taxa used in this study.

Taxon	Voucher	Tissues	Locality	Coordinates	City, State	Country
Ichthyoelephas humeralis	LBP 19326	76121	Río Guayas basin	−1.796694, −79.533611	Babahoyo, Los Ríos	Ecuador
Ichthyoelephas humeralis	LBP 19326	76122	Río Guayas basin	−1.796694, −79.533611	Babahoyo, Los Ríos	Ecuador
Ichthyoelephas longirostris	ANSP 192865	6609	Río Magdalena basin	5.2056389, −75.7471389	Honda, Tolima	Colombia
Prochilodus argenteus	LBP 251	4216	Rio São Francisco basin	−18.1965278, −45.2356944	Três Marias, Minas Gerais	Brazil
Prochilodus argenteus	LBP 251	4217	Rio São Francisco basin	−18.1965278, −45.2356944	Três Marias, Minas Gerais	Brazil
Prochilodus brevis	LBP 2496	16385	Açude Araçá, northeastern Brazilian drainage	−5.8683333, −35.3550000	Macaíba, Rio Grande do Norte	Brazil
Prochilodus brevis	LBP 2496	16386	Açude Araçá, northeastern Brazilian drainage	−5.8683333, −35.3550000	Macaíba, Rio Grande do Norte	Brazil
Prochilodus britskii	LBP 20269	79757	Rio Apiacás, Tapajós, Amazon basin	−10.350194, −56.982417	Alta Floresta, Mato Grosso	Brazil
Prochilodus britskii	LBP 20269	79758	Rio Apiacás, Tapajós, Amazon basin	−10.350194, −56.982417	Alta Floresta, Mato Grosso	Brazil
Prochilodus costatus	LBP 252	4222	Rio São Francisco basin	−18.1965278, −45.2571389	Três Marias, Minas Gerais	Brazil
Prochilodus costatus	LBP 252	4223	Rio São Francisco basin	−18.1965278, −45.2571389	Três Marias, Minas Gerais	Brazil
Prochilodus harttii	LBP 7211	33175	Rio Pardo, eastern Brazilian drainage	−15.5218350, −41.5049500	Machado Mineiro, Minas Gerais	Brazil
Prochilodus harttii	LBP 7211	33176	Rio Pardo, eastern Brazilian drainage	−15.5218350, −41.5049500	Machado Mineiro, Minas Gerais	Brazil
Prochilodus lacustris	LBP 9104	42731	Rio Poti, Parnaíba basin	−5.0574444, −42.8084722	Teresina, Piauí	Brazil
Prochilodus lacustris	LBP 9104	42732	Rio Poti, Parnaíba basin	−5.0574444, −42.8084722	Teresina, Piauí	Brazil
Prochilodus lineatus	LBP 45	3611	Rio Miranda, Paraguay basin	−19.5684167, −57.0200833	Corumbá, Mato Grosso do Sul	Brazil
Prochilodus lineatus	LBP 2348	16071	Rio Paraíba do Sul, eastern Brazilian drainage	−22.0000000, −41.3333333	Campos dos Goytacazes, Rio de Janeiro	Brazil
Prochilodus magdalenae	GR-93-1	GR207	Río Magdalena basin	9.3611100, −74.7316600	Magangue, Bolívar	Colombia
Prochilodus magdalenae	GR-93-1	GR208	Río Magdalena basin	9.3611100, −74.7316600	Magangue, Bolívar	Colombia
Prochilodus mariae	LBP 2188	15561	Laguna de Castilleros, Río Orinoco basin	7.5141389, −66.1555000	Caicara del Orinoco, Bolivar	Venezuela
Prochilodus mariae	LBP 2188	15562	Laguna de Castilleros, Río Orinoco basin	7.5141389, −66.1555000	Caicara del Orinoco, Bolivar	Venezuela
Prochilodus nigricans	LBP 7841	36858	Rio Araguaia, Amazon basin	−13.3166667, −50.6166667	Cocalinho, Mato Grosso	Brazil
Prochilodus nigricans	LBP 8589	43397	Rio Arinos/Tapajós, Amazon basin	−14.1541389, −56.0948889	Diamantino, Mato Grosso	Brazil
Prochilodus nigricans	LBP 8589	43398	Rio Arinos/Tapajós, Amazon basin	−14.1541389, −56.0948889	Diamantino, Mato Grosso	Brazil
Prochilodus nigricans	LBP 12865	53496	Rio Tapajós, Amazon basin	−4.8705833, −56.8552778	Itaituba, Pará	Brazil
Prochilodus nigricans	LBP 1690	12754	Rio Amazonas, Amazon basin	−3.0772222, −59.8255556	Manaus, Amazonas	Brazil
Prochilodus nigricans	OS 18792	PE10045	Río Itaya, Amazon basin	−3.7898610, −73.2495830	Maynas, Loreto	Peru
Prochilodus nigricans	OS 18792	PE10058	Río Itaya, Amazon basin	−3.7898610, −73.2495830	Maynas, Loreto	Peru
Prochilodus nigricans	FMNH 113534	T54	Rio Itaya, Amazon basin	−3.7719444, −73.2377778	Iquitos, Loreto	Peru
Prochilodus reticulatus	LBP 6127	29513	Río Catatumbo, Lago Maracaibo	9.0856389, −72.2306944	Encontrados, Zulia	Venezuela
Prochilodus reticulatus	LBP 6127	29514	Río Catatumbo, Lago Maracaibo	9.0856389, −72.2306944	Encontrados, Zulia	Venezuela
Prochilodus cf. *rubrotaeniatus*	ANSP 40692	P4313	upper Río Orinoco basin	3.1637222, −65.6861389	La Esmeralda, Amazonas	Venezuela
Prochilodus rubrotaeniatus	MHNG 2705.008	SU07108	Rivière Paikali, Corantijn basin	2.3460600, −56.8320000	Kwamalasamutu, Sipaliwini	Suriname
Prochilodus rubrotaeniatus	MHNG 2717.017	SU08776	Tapanahony River, Marowijne basin	3.3660000, −55.4321000	Palumeu, Sipaliwini	Suriname
Prochilodus rubrotaeniatus	USNM 403693	GY11461	Cuyuni River, Essequibo basin	6.8098100, −59.7986500	Cuyuni-Mazaruni	Guyana
Prochilodus vimboides	LBP 2349	16011	Rio Doce, eastern Brazilian drainage	−19.4096111, −40.0652222	Sooretama, Espírito Santo	Brazil
Prochilodus vimboides	LBP 10180	47662	Rio Mucuri, eastern Brazilian drainage	−17.6951111, −40.7698056	Carlos Chagas, Minas Gerais	Brazil
Semaprochilodus brama	LBP 12776	41019	Rio Araguaia, Amazon basin	−13.3103611, −50.6132222	Cocalinho, Mato Grosso	Brazil
Semaprochilodus brama	LBP 12807	41171	Rio Araguaia, Amazon basin	−13.3103611, −50.6132222	Cocalinho, Mato Grosso	Brazil
Semaprochilodus insignis	LBP 1692	12761	Rio Amazonas, Amazon basin	−3.0772222, −59.8255556	Manaus, Amazonas	Brazil
Semaprochilodus insignis	LBP 1692	12762	Rio Amazonas, Amazon basin	−3.0772222, −59.8255556	Manaus, Amazonas	Brazil
Semaprochilodus insignis	OS 18380	PE10001	Río Itaya, Amazon basin	−4.2258000, −73.4850000	Maynas, Loreto	Peru
Semaprochilodus insignis	ANSP 180205	T43	Río Nanay, Amazon basin	−3.7800000, −73.3426389	Iquitos, Loreto	Peru

Table 1 (continued)

Taxon	Voucher	Tissues	Locality	Coordinates	City, State	Country
Semaprochilodus kneri	LBP 1384	12734	Río Orinoco basin	7.6416667, −66.1500000	Caicara del Orinoco, Bolivar	Venezuela
Semaprochilodus kneri	LBP 3041	19139	Río Orinoco basin	7.6365556, −66.3178333	Caicara del Orinoco, Bolivar	Venezuela
Semaprochilodus kneri	LBP 3041	19140	Río Orinoco basin	7.6365556, −66.3178333	Caicara del Orinoco, Bolivar	Venezuela
Semaprochilodus kneri	ANSP 187277	P4298	Río Apure, Orinoco basin	7.7027778, −66.9611111	San Fernando de Apure, Apure	Venezuela
Semaprochilodus laticeps	LBP 1383	12727	Río Orinoco basin	7.6416667, −66.1500000	Caicara del Orinoco, Bolivar	Venezuela
Semaprochilodus laticeps	LBP 1383	12728	Río Orinoco basin	7.6416667, −66.1500000	Caicara del Orinoco, Bolivar	Venezuela
Semaprochilodus laticeps	FMNH 113712	2004BSAQ01	Aquarium specimen purchased in Chicago	Unknown	Unknown	Unknown
Semaprochilodus taeniurus	LBP 1691	12757	Rio Amazonas, Amazon basin	−3.0772222, −59.8255556	Manaus, Amazonas	Brazil
Semaprochilodus taeniurus	LBP 1691	12758	Rio Amazonas, Amazon basin	−3.0772222, −59.8255556	Manaus, Amazonas	Brazil
Semaprochilodus taeniurus	LBP 1691	12759	Rio Amazonas, Amazon basin	−3.0772222, −59.8255556	Manaus, Amazonas	Brazil
Semaprochilodus varii	MHNG uncatalogued	15729	Tapanahony River, Marowijne basin	3.1983333, −55.4075000	Sipaliwini	Suriname
Semaprochilodus varii	ANSP 187435	6929	Lawa River, Marowijne basin	3.3252778, −54.0633333	Sipaliwini	Suriname
Leporellus cf. *vittatus*	AUM 54212	T09912	Río Cataniapo, Orinoco basin	5.53375, −67.37395	Amazonas	Venezuela
Leporinus desmotes	AUM 43700	V5274	Rio Casiquiare	2.1557, −66.46377	Amazonas	Venezuela
Leporinus friderici	ANSP 189264	7015	Lawa river, Marowijne basin	3.325278, −54.063333	Sipaliwini	Suriname
Leporinus striatus	LBP 3180	16871	Rio Paranapanema, upper Paraná basin	−23.33333, −48.566667	Itatinga, São Paulo	Brazil
Abramites hypselonotus	AUM 53775	T08985	Rio Guanare, Orinoco basin	8.91411, −69.7611	Guanare, Portuguesa	Venezuela
Schizodon scotorhabdotus	AUM 53654	T09707	Río Manapiare, Orinoco basin	5.33714, −66.05146	Amazonas	Venezuela
Chilodus fritillus	AUM 51355	T10201	Río Madre de Dios	−12.2771300, −69.1523700	Madre de Dios	Peru
Caenotropus mestomorgmatos	ANSP 180516	T48	Río Nanay, Amazon basin	−3.7791667, −76.7666667	Iquitos, Loreto	Peru
Curimatopsis macrolepis	ANSP 178188	1697	Río Nanay, Amazon basin	−3.7563889, −73.2911111	Iquitos, Loreto	Peru
Curimata cyprinoides	USNM 402471	GY11-1-03	Cuyuni river, Essequibo basin	6.84722, −60.1363	Cuyuni-Mazaruni	Guyana
Psectrogaster amazonica	OS 18313	PE10113	Río Amazonas, Amazon basin	−3.7182778, −73.2128056	Iquitos, Loreto	Peru
Cyphocharax gilbert	LBP 8343	40130	Rio Mucuri, eastern Brazilian drainage	−17.695111, −40.769806	Carlos Chagas, Minas Gerais	Brazil
Cyphocharax spilotus	LBP 4747	25521	Rio Guaíba, southern Brazilian drainage	−30.285278, −51.300278	Barra do Ribeiro, Rio Grande do Sul	Brazil
Anodus elongatus	OS 18724	PE10110	Río Amazonas, Amazon basin	−3.7182778, −73.2128056	Iquitos, Loreto	Peru
Hemiodus unimaculatus	OS18345	PE10076	Río Nanay, Amazon basin	−3.7516670, −73.3162500	Iquitos, Loreto	Peru
Apareiodon affinis	LBP 4591	24665	Rio Paranapanema, Paraná basin	−22.9042778, −50.0010556	Salto Grande, São Paulo	Brazil
Parodon nasus	LBP 1135	5635	Rio Tietê, Paraná basin	−22.8666667, −48.3833333	Botucatu, São Paulo	Brazil
Colossoma macropomum	LBP 5173	26648	Rio Amazonas	−1.305556, −48.607778	Belém, Pará	Brazil
Catoprion mento	LBP 7556	35624	Rio Cuiabá, upper Paraguay basin	−16.194306, −55.806972	Barão de Melgaço, Mato Grosso	Brazil
Metynnis lippincottianus	LBP 6282	29688	Rio Grande, upper Paraná basin	−20.947083, −48.14775	Pontal, São Paulo	Brazil
Myleus schomburgkii	OS 18990	PE10044	Río Nanay, Amazon basin	−3.7516670, −73.3162500	Iquitos, Loreto	Peru
Brycon pesu	OS 18361	PE10072	Río Nanay, Amazon basin	−3.7516670, −73.3162500	Iquitos, Loreto	Peru

sequences of the nuclear *myosin heavy chain 6 gene* (Myh6, 711 bp), *recombination activating gene 1* (Rag1, 1379 bp), and *recombination activating gene 2* (Rag2, 1030 bp) through nested-PCR following Oliveira et al. (2011). Full primer sequences and their sources appear in the accompanying Data in Brief (see Table 2 in Frable et al., submitted for publication).

Amplification reactions had a total volume of 12.5 µl, containing 9.075 µl of double-distilled water, 1.25 µl 5× reaction buffer, 0.375 $MgCl_2$, 0.25 µl dNTP mix at 8 mM, 0.25 µl of each primer at 10 µM, 0.05 µl Platinum Taq DNA polymerase enzyme (Invitrogen; www.invitrogen.com) and 1.0 µl genomic DNA (10–50 ng). The PCR consisted of an initial denaturation (4 min at 95 °C) followed by 28–30 cycles of chain denaturation (30 s at 95 °C), primer hybridization (30–60 s at 52–54 °C), and nucleotide extension (30–60 s at 72 °C).

After visualization of the fragments using 1% agarose gel, we sequenced using dye terminators (BigDye™ Terminator v 3.1 Cycle Sequencing Ready Reaction Kit, Applied Biosystems) purified again through ethanol precipitation. We sequenced the samples on an ABI 3130-Genetic Analyzer (Applied Biosystems) at either Oregon State University (Corvallis, Oregon, USA) or Universidade Estadual Paulista (Botucatu, São Paulo, Brazil).

2.3. Alignment and phylogenetic analyses

After sequencing, we assembled and edited consensus sequences in Geneious 7.1.7 (Kearse et al., 2012; www.geneious.com) and applied IUPAC ambiguity codes where we detected uncertainty of nucleotide identity. We aligned the consensus
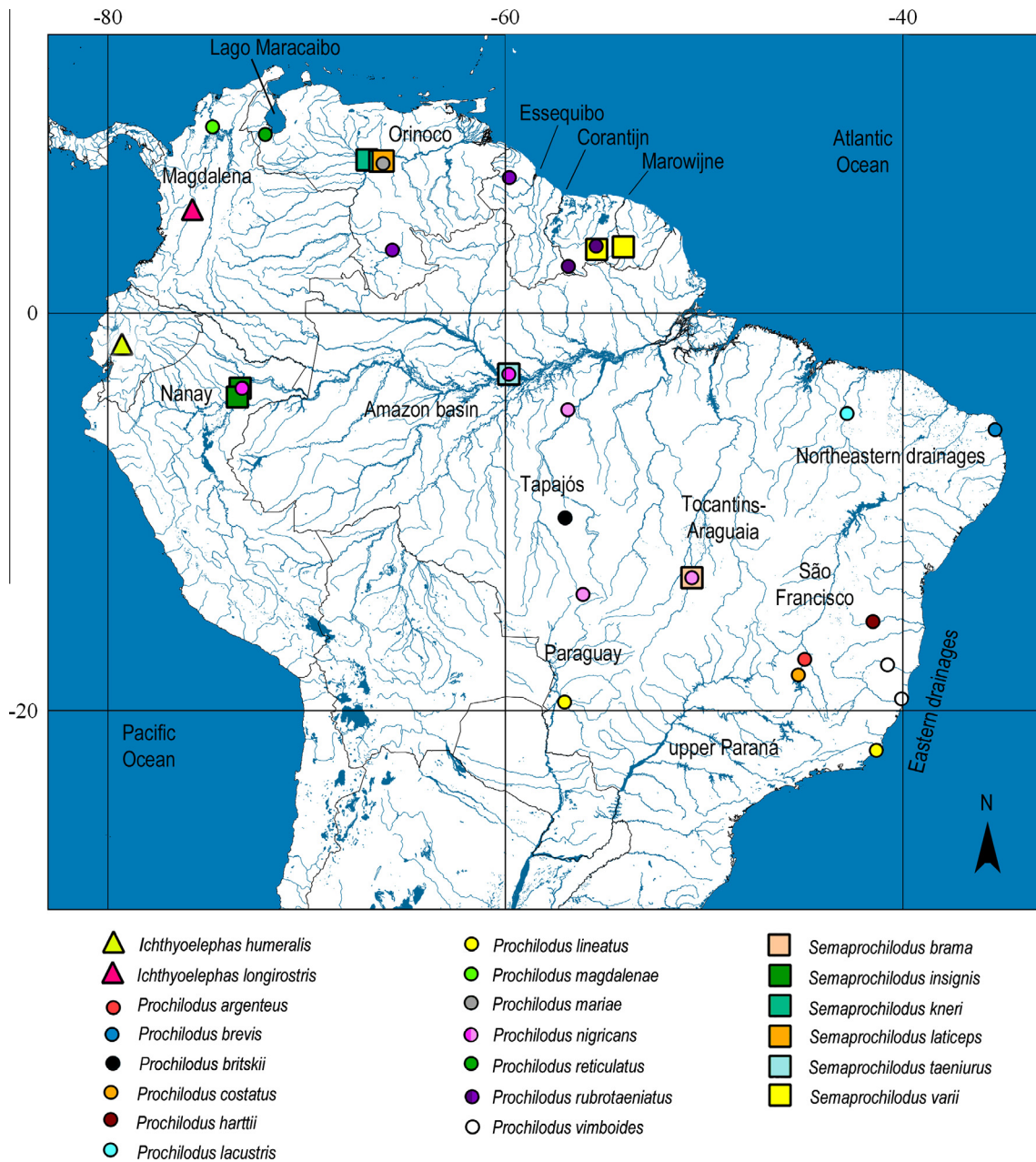
Fig. 3. Central and northern South America showing voucher localities for specimens of Prochilodontidae used in this study.

sequences of each gene using the Muscle algorithm (Edgar, 2004) as implemented in Geneious and inspected alignments by eye. To evaluate the occurrence of substitution saturation, we estimated the index of substitution saturation (Iss) using Dambe 5.3.38 (Xia, 2013). Preliminary analysis of the complete 16S data revealed many uncertain alignments due to length polymorphism in loop regions. These hypervariable regions were excluded in a reduced 16S submatrix that was in turn concatenated with the other five genes. PartitionFinder 1.1.0 (Lanfear et al., 2012) was used to select the best partitioning scheme and the best-fit model of molecular evolution for each subset in that scheme using the Bayesian information criterion (BIC); we assumed 16 possible partitions, one for each codon position in the five coding genes, plus the 16S stems (Frable et al., submitted for publication).

Bayesian analysis of the partitioned matrix using MrBayes 3.2 (Ronquist et al., 2012) included assignation of the chosen models for each partitioning scheme. We performed two runs of four

independent MCMC chains with 20 million replicates each, sampling every two thousand generations. Stationarity and sufficient mixing (ESS > 200) were checked using Tracer 1.6 (Rambaut et al., 2014). The first 10% of the generations were discarded as burn-in in TreeAnnotator, and the remaining 9001 trees were summarized in a phylogram with mean branch lengths and a maximum credibility tree with posterior probabilities for each split. The final tree was visualized and edited with FigTree v1.4.2.

Maximum likelihood (ML) analyses were performed in RAxML HPC v.8 on XSEDE (Stamatakis, 2006) as implemented on the CIPRES Scientific Gateway v.3.3 (Miller et al., 2010). The analysis employed the partitioning scheme identified by PartitionFinder with *Brycon pesu* as the outgroup and rapid bootstrapping performed simultaneously with the search for the best-scoring tree. All other parameters were left at default values.

We implemented *BEAST to reconstruct consensus species tree with informed priors on substitutions and rates of evolution

Table 2
Results of Shimodaira-Hasegawa tests of alternative morphological hypotheses from Castro and Vari (2004) with and without optimization of base frequencies and rate matrices in phangorn. Marginal *P*-values near alpha = 0.05 are marked with a single asterisk. Double asterisks indicate *P*-values far below alpha = 0.05 and statistical rejection of topological equivalence.

Constraint	No optimization			Optimized		
	ln L	∂L	P	ln L	∂L	P
None	−45938.14	0.00	0.9262	−43202.76	0.00	0.9346
1. *Semaprochilodus taeniurus*	−45945.25	7.11	0.7194	−43209.81	7.54	0.7003
2. *Semaprochilodus*	−45950.96	12.82	0.6049	−43215.68	13.42	0.5743
3. *Prochilodus*	−46004.41	66.27	0.0520*	−43264.47	62.21	0.0501*
4. *Ichthyoelephas*	−46128.42	190.27	0.0000**	−43375.73	173.47	0.0000**

(Heled and Drummond, 2010). Tips were assigned to nominal species. Given the non-monophyletic nature of *Prochilodus nigricans* and *P. rubrotaeniatus* in the concatenated analysis, we assigned those species to two separate operational taxonomic units (OTUs). Given the overwhelming morphological support for the monophyly of Prochilodontidae (Castro and Vari, 2004), we enforced the monophyly of the family for the *BEAST analysis. Parameters and priors for the *BEAST analysis were configured using BEAUTi 1.8.3 (Drummond et al., 2012) (see Table 5 in Frable et al., submitted for publication). We used the BIC in PartitionFinder 1.1.4 to determine the best partitioning scheme for substitution models for each gene (see Table 4 in Frable et al., submitted for publication) and implemented the uncorrelated lognormal distribution (UCLN) rate variation model to estimate trees in BEAST v 1.8.3. Four independent MCMC were run for 250 million generations, sampling every 25,000 generations. Convergence was evaluated in Tracer v. 1.6 (Rambaut et al., 2014) using effective sample size (ESS), prior convergence and likelihood (−ln L) of the priors and posterior estimates after removal of a 20% burn-in. Further detail of the *BEAST analysis and prior settings are reported in Frable et al. (submitted for publication).

To test the support for the molecular topology versus the morphological hypothesis of Castro and Vari (2004), we compared the ML unconstrained tree to the ML trees generated in RAxML under four different constraints. The first forces *Semaprochilodus taeniurus* as sister to a clade containing *S. kneri* and *S. insignis*. Constraint 2 adds a monophyly constraint to *S. insignis*, thereby fully matching the morphological phylogeny given for that genus. The third forces monophyly of all clades within *Prochilodus* given in Castro and Vari (2004), but does not constrain arrangements within those clades, nor force monophyly of species. Constraint 4 forces *Ichthyoelephas* to be sister to *Semaprochilodus*. Constraint trees were constructed in Mesquite 3.04 (Maddison and Maddison, 2013). We used the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) as implemented in phangorn v2.0.1 (Schliep, 2011) to compare the maximum likelihoods inferred under these scenarios. Within phangorn, we compared the likelihood fits assuming a GTR substitution model, four discrete intervals of the gamma distribution (k = 4), and 10,000 bootstrap replicates. We performed analyses with and without optimizing the rate matrix and base frequencies (Table 2).

3. Results

The concatenated matrix included 5279 bp spanning all recognized species of the Prochilodontidae and six closely related families in the Characiformes. Missing data correspond to 8.6% of the total matrix. Of the 1970 variable sites, 1463 were parsimony-informative. Nucleotide frequencies appear in Frable et al. (submitted for publication). The Iss values indicated no substitution saturation in transitions or transversions in both symmetrical and asymmetrical topologies. PartitionFinder segregated the 16 possible subsets into six partitions and selected their best-fit

model of nucleotide evolution (see Table 3 in Frable et al., submitted for publication). Generated sequences are deposited in GenBank with accession numbers KX086740–KX087100 and available in Frable et al. (submitted for publication).

Both Bayesian (Fig. 4) and likelihood (Frable et al., submitted for publication) analyses of the concatenated matrix strongly support the monophyly of the Prochilodontidae as well as the monophyly of all three genera. Our phylogeny recovers for the first time *Ichthyoelephas* as sister to the clade containing *Prochilodus* and *Semaprochilodus*, contrasting with the morphological hypothesis (Castro and Vari, 2004) that placed *Prochilodus* as the sister to a clade containing *Ichthyoelephas* and *Semaprochilodus*. The SH test strongly rejects this morphological placement of *Ichthyoelephas* (Table 2; see also Frable et al., submitted for publication, for the maximum likelihood topology reconstructed under this constraint).

The arrangement of species within a monophyletic *Semaprochilodus* disagrees slightly with the morphological hypothesis (Castro and Vari, 2004) by placing *S. taeniurus* from the Amazon basin sister to all other species instead of to the clade containing only *S. insignis* from the Amazon basin and *S. kneri* of the Orinoco basin, and by recovering a non-monophyletic *S. insignis*. SH tests, however, fail to reject either morphological hypothesis (Table 2; see also Frable et al., submitted for publication, for images of the compared topologies). Our results resolve Castro and Vari's (2004) trichotomy of the remaining three species by placing *S. brama* from the Araguaia basin as sister to a clade containing *S. laticeps* of the Orinoco basin and *S. varii* from the Marowijne River of Suriname.

We obtained a pectinate phylogeny (Fig. 4) among the species of *Prochilodus* that agrees largely with the morphological topology (Fig. 2; Castro and Vari, 2004), and provides substantially more interspecific resolution. In the molecular topology, *P. vimboides* from the Rio Doce and Rio Mucuri in the eastern Brazil is sister to all remaining species of *Prochilodus*. West of the Andes, *P. magdalenae* from the Río Magdalena in Colombia forms a well-supported clade with *P. reticulatus* from Lago Maracaibo in Venezuela, although we did not obtain reciprocal monophyly of the two putative species. Within a clade containing the remaining ten *Prochilodus* species, *P. mariae* from the Río Orinoco appears as sister to the rare and geographically restricted *P. britskii* from the upper Rio Tapajós, albeit with low Bayesian and likelihood support for that relationship. This placement of *P. mariae* conflicts with the morphological hypothesis (see Section 4). We resolve a strongly supported sister relationship between *P. argenteus*, endemic to the Rio São Francisco, and *P. harttii* of the Rio Pardo and Rio Jequitinhonha of eastern Brazil, which is, in turn, sister to a clade formed by the six remaining species of *Prochilodus*. In that clade, *P. lineatus* of the Paraguay and Paraíba do Sul basins is nested within a paraphyletic *P. costatus* from the Rio São Francisco. That clade is, in turn, sister to a complex group of several species embedded within the widespread Amazonian *P. nigricans*.

Within that complex, which we herein call the "*Prochilodus nigricans* group" (Fig. 4), we found two distinct lineages. The first
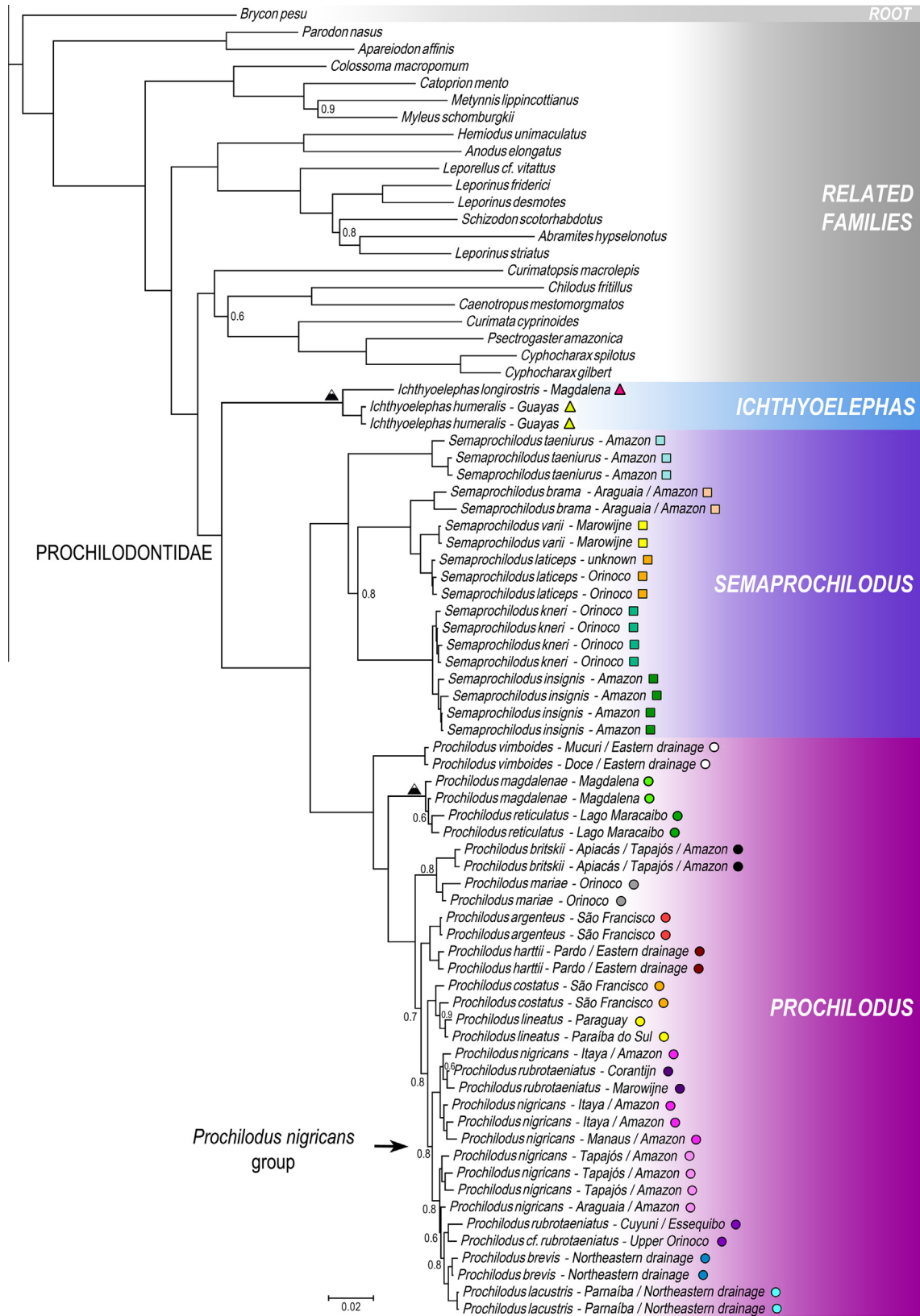
Fig. 4. Phylogenetic relationships of Prochilodontidae based on a Bayesian analysis of the concatenated dataset. Clades without numbers are supported with Bayesian posterior probabilities ⩾0.95 and numbered clades have lower posterior probabilities. Colored symbols correspond to those in Fig. 2. Mountain symbols indicate trans-Andean clades. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

well-supported clade is formed by *P. nigricans* from the mainstream Amazon basin plus *P. rubrotaeniatus* from the Corantijn and Marowijne river basins in Suriname. The second contains *P.*

nigricans from the eastern Amazonian tributaries (Rio Araguaia and Rio Tapajós) grouped with specimens tentatively assigned to *P. rubrotaeniatus* from the upper Río Orinoco (*P.* cf. *rubrotaeniatus*

in Fig. 4), *P. rubrotaeniatus* from the Cuyuni-Essequibo River in Guyana, and a well-supported group formed by *P. brevis* plus *P. lacustris*, both from northeastern Brazil.

Castro and Vari (2004) also recognized this cluster of four species on the basis of a single morphological synapomorphy (presence of chevron-shaped dark bars on the caudal fin), albeit with the inclusion of *Prochilodus mariae*, which demonstrates similar coloration. The most likely molecular tree with those five species constrained to monophyly (Frable et al., submitted for publication) is substantially less likely than the unconstrained topology, which places *Prochilodus mariae* as sister to *P. britskii*, though the comparison narrowly misses significance at alpha = 0.05 in the SH tests (Table 2).

The maximum clade credibility species tree from four independent *BEAST runs yielded generally high (>0.6) posterior probabilities for inferred relationships within Prochilodontidae (Fig. 5). Species relationships within the *Prochilodus nigricans* group exhibited lower posterior supports reflecting the poor resolution in the previous analyses. The topology of the species tree is generally congruent with the ML and Bayesian phylogenies (Fig. 4) with the exception of the placement of *Prochilodus mariae + P. britskii* sister to the *Prochilodus nigricans* group rather than sister to the

group containing *P. costatus + P. lineatus* and *P. harttii + P. argenteus* (Fig. 5). However, support for *P. mariae* and *P. britskii* as sister to the "*Prochilodus nigricans* group" is very low (posterior probability = 0.37). The lower support for the placement of *P. britskii* and for the sister relationship of the two *Ichthyoelephas* species may result from missing data (see Frable et al., submitted for publication).

While this study was not designed to infer interfamilial relationships, the scheme of relationships in the related families largely match those found in recent molecular studies (Oliveira et al., 2011; Melo et al., 2014). The close relationship between Chilodontidae, Curimatidae and Prochilodontidae, is of particular note, given that it is recovered in all molecular analyses to date, but conflicts with the sister relationship between Chilodontidae and Anostomidae strongly supported by osteological analyses (Vari, 1983; Dillman et al., 2015). Surprisingly, we also found a strongly supported relationship between Hemiodontidae and Anostomidae and of the clade formed by those two families as sister to Chilodontidae, Curimatidae and Prochilodontidae along with an unexpected placement of *Curimatopsis* as sister to Chilodontidae plus Curimatidae under the Bayesian topology (but not in the ML tree). *Curimatopsis* is sister to all other members of Curimatidae
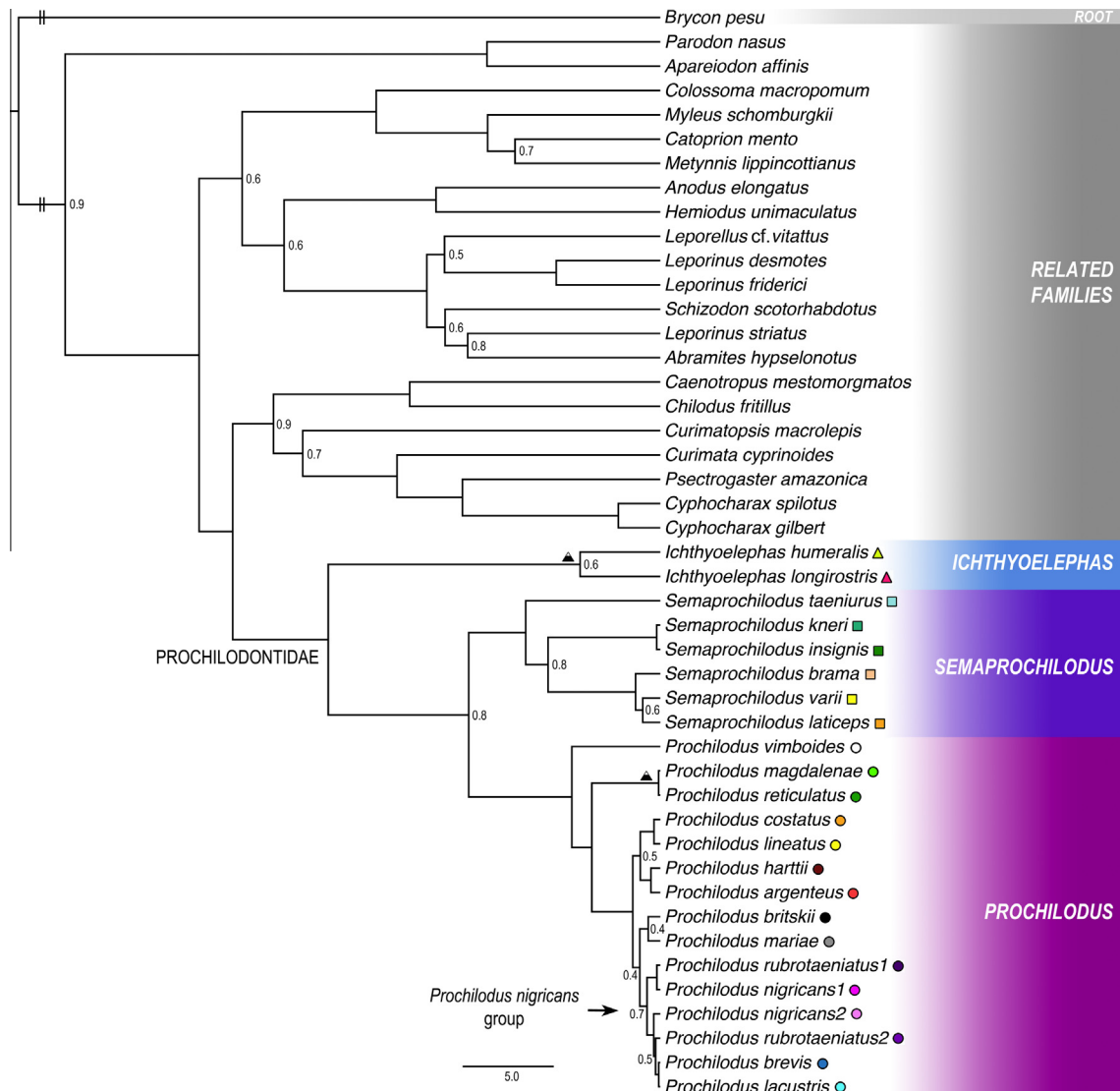

Fig. 5. Species tree for Prochilodontidae inferred by *BEAST. Posterior probabilities between 0.6 and 0.9 (inclusive) are shown. Unlabelled nodes received posterior probabilities in excess of 0.9. Mountain symbols indicate trans-Andean clades.

on the basis of numerous morphological synapomorphies. Further study is necessary to determine whether these arrangements reflect biological reality or are simply artifacts of limited taxon sampling of closely related taxa and examples of long-branch attraction.

4. Discussion

4.1. Monophyly of the Prochilodontidae and intergeneric relationships

Our molecular-based analysis corroborates Vari's (1983) and Castro and Vari's (2004) hypothesis of the monophyly of the Prochilodontidae which was supported by 58 morphological synapomorphies. Dillman et al. (2015) reanalyzed a supermatrix of all morphological characters proposed as informative within Anostomoidea and their results slightly reduced the support for the Prochilodontidae to 56 synapomorphies. We reconstruct Prochilodontidae as sister to a clade composed by Chilodontidae plus Curimatidae, a grouping of three families recovered in other recent molecular phylogenies (Oliveira et al., 2011; Melo et al., 2014) but which runs counter to the hypothesis of relationships arrived at by morphology-based analyses (e.g. Vari, 1983; Castro and Vari, 2004; Dillman et al., 2015), which place Chilodontidae sister to Anostomidae. All three molecular studies used the same combination of mitochondrial loci useful for resolving shallow clades and nuclear coding genes best for resolving deep clades. As such, the concordance of the results among these studies is expected. Our labs are actively working to test these relationships with other molecular markers.

Our results strongly support Castro and Vari's (2004) hypothesis of monophyly for each prochilodontid genus, *Ichthyoelephas*, *Prochilodus* and *Semaprochilodus*, based on 32, 16 and 13 morphological synapomorphies, respectively (Castro and Vari, 2004). In contrast to the morphological result, our results yield a novel, strongly supported sister relationship between *Ichthyoelephas* and a clade consisting of *Prochilodus* and *Semaprochilodus* (Fig. 4). Castro and Vari (2004) conversely proposed a sister relationship between *Ichthyoelephas* and *Semaprochilodus* based on eight synapomorphies (their synapomorphies 78–85), with this clade sister to *Prochilodus*, suggesting substantial conflict between the two results. However, re-examination of the morphological matrix reveals that the conflict is less severe than might be imagined at first consideration.

Synapomorphies 78, 81 and 82 of Castro and Vari (2004) have equally parsimonious optimizations on the molecular topology due the lack of the respective features (a maxillary foramen, a ridge on the ventral margin of the replacement tooth trench of the dentary, and an articular facet of the interopercle) in non-prochilodontids. The molecular results simply alter the reconstructed polarity of the state changes for these characters. For example, the large form of the maxillary foramen optimized as a synapomorphic for *Semaprochilodus* and *Ichthyoelephas* under the morphological hypothesis (synapomorphy 78) becomes a plesiomorphy on the molecular topology. The smaller form of the opening in turn becomes synapomorphic for *Prochilodus*.

Castro and Vari (2004) interpreted character 10 as a homoplasy and listed it as their synapomorphy 3 for the family, with subsequent reduction in *Ichthyoelephas*. We instead optimize it as a homology on the molecular topology. The morphology in question concerns hypertrophy of the fourth upper pharyngeal toothplate such that it completely surrounds the dorsal surface of the first infrapharyngobranchial: a condition to our knowledge found only in *Prochilodus* and *Semaprochilodus* among all characiforms. This morphological character therefore supports the molecular hypothesis.

Those same authors considered their character 121 as a homoplasy with uncertain optimization due to the possession of a shared character state in *Semaprochilodus* and *Prochilodus* (a bifurcate procumbent dorsal fin spine), a unique undivided morphology in *Ichthyoelephas*, and the absence of the spine in more distantly related taxa. The optimization of this character is still uncertain on the molecular topology, and either arrangement is equally parsimonious if the character states are considered unordered and unpolarized. However, the molecular topology suggests an arguably more intuitive polarization in which the simpler, undivided form of the spine is plesiomorphic, while the more complicated divided form is apomorphic.

The derived state of character 116 (Castro and Vari, 2004), concerning the presence of dark wavy lines on the body surface, occurs in nine species of *Prochilodus* and all species of *Semaprochilodus*, but is absent in *Ichthyoelephas* and in most non-prochilodontid characiforms. This character is homoplasious in either the molecular or the morphological reconstructions, and the alternative placements of *Ichthyoelephas* do not affect its optimization within *Prochilodus*. This character is therefore equivocal with respect to intergeneric relationships in Prochilodontidae.

Five of Castro and Vari's proposed synapomorphies for the *Ichthyoelephas* and *Semaprochilodus* clade do optimize as homoplasies within the context of our phylogeny. These are synapomorphies 79 (presence of an articular facet on the dorsal margin of the maxilla), 80 (presence of a process on the dorsal margin of the maxilla), 83 (the wide form of the lateral ethmoid), 84 (the reduction or loss of a process of the orbitosphenoid) and 85 (presence of fleshy flaps on the middorsal scales). Thus, the molecular hypothesis would imply either independent acquisition or secondary loss of these characters.

In summary, the conflict between the datasets is not extreme. The molecular placement of *Ichthyoelephas* implies a morphological optimization that is only four steps less parsimonious than the previous hypothesis. In the light of the very strong support for the molecular placement, the only slightly less parsimonious morphological solution represented by the molecular topology, and the presence of a uniquely shared morphology of the fourth upper pharyngeal tooth plate in *Semaprochilodus* and *Prochilodus*, the molecular placement of *Ichthyoelephas* likely represents biological reality.

4.2. Interspecific relationships within Semaprochilodus

Castro and Vari (2004) proposed *Semaprochilodus taeniurus* as sister to a clade formed by *S. insignis* plus *S. kneri* based on two synapomorphies related to the epibranchials and the laterosensory canal system (their synapomorphies 100 and 101, respectively). Both features appear only in these three taxa within Prochilodontidae. Thus, our phylogenetic hypothesis based on both Bayesian (Fig. 4) and ML analyses (Frable et al., submitted for publication), which recovered *S. taeniurus* as sister to the clade formed by all remaining *Semaprochilodus* species, implies homoplasy in these characters. Further molecular phylogenies incorporating more loci will likely resolve the actual intrageneric placement of *S. taeniurus* with greater confidence.

The placement of *Semaprochilodus taeniurus* aside, all morphological and molecular analyses agree closely on the relationships of the remaining species of *Semaprochilodus*. For example, Castro and Vari (2004) first hypothesized a close relationship between *S. kneri* of the Orinoco basin and *S. insignis* from the Amazon basin based on the loss of the suprapreopercle and the elaboration of the maxilla at the attachment point of the primordial ligament (their synapomorphies 102 and 103). We confirmed the close relationship of these species and indeed, discovered very little

molecular differentiation between them. These two species are quite similar morphologically with only few meristic nuances distinguishing them. Future studies should test their taxonomic validity.

We recovered a clade composed by *Semaprochilodus brama, S. laticeps* and *S. varii* that was previously hypothesized by Castro and Vari (2004) on the basis of the derived presence of "very dark pigmentation on the border of the operculum and the adjoining region of cleithrum". Those authors were unable, however, to resolve the relationships among them. Our results demonstrate that *S. laticeps* from the Río Orinoco basin and *S. varii* from the Marowijne River of the Atlantic slope of Suriname are most closely related. *Semaprochilodus brama* from the Rio Araguaia (along possibly with populations of that species from the Rio Xingu not examined herein) is sister to the clade composed by those two species.

4.3. Phylogenetic relationships within Prochilodus

In their morphological study of *Prochilodus*, Castro and Vari (2004) proposed three synapomorphies for subclades involving color pattern and squamation. Given that paucity of information, molecular approaches return greater phylogenetic resolution, but not at all certain that the phylogenies would be congruent. Nevertheless, two of the three clades within *Prochilodus* recognized by Castro and Vari (2004) appear in our reconstruction, and we reconstruct the species in the root polytomy of the earlier paper as successive sister groups to the remainder of the family.

Prochilodus vimboides, a morphologically distinct species from the Rio Doce and Rio Mucuri in eastern Brazil, is sister to all the remaining species of *Prochilodus*. Turning to the remaining species in the genus, we recovered the first evidence of a close relationship between the species from west of Andes, *P. magdalenae* and *P. reticulatus*, although similarities between these forms have long been noted. Steindachner (1879) first observed the similarities in his description of *P. asper* var. *magdalenae* (=*P. magdalenae*) for what he considered a trans-Andean Colombian population of *P. asper* (the latter species is currently considered a synonym of *P. reticulatus*). Though *P. magdalenae* and *P. reticulatus* differ in the ranges and modal numbers of scales and vertebrae and have allopatric distributions (Castro and Vari, 2004), the lack of molecular differentiation results in a paraphyletic *P. magdalenae* in our analysis. The substantial morphological and molecular similarities between these species and the limited scale of their morphological differences suggest that they may represent only one species with perhaps allopatric differentiation resulting from the uplift of the Sierra del Perijá which separates the populations. The placement of this species pair is consistent with an earlier phylogeny using the mtDNA control region (Sivasundar et al., 2001) which found *P. magdalenae* as sister to *P. mariae, P. nigricans* and *P. lineatus* (*P. reticulatus* not examined).

Castro and Vari (2004) considered *Prochilodus mariae* to fall within a clade also containing *P. brevis, P. lacustris, P. nigricans* and *P. rubrotaeniatus* due to the derived "presence of two to eight irregular, vertical, somewhat chevron-shaped, dark bars on the caudal fin". That set of relationships was not retrieved in our analysis, in which the two specimens of *P. mariae* from the lower Río Orinoco in Venezuela appeared as sister to *P. britskii* from the upper Rio Tapajós, albeit with low support. The nearly significant log likelihood difference between the unconstrained molecular tree and that produced with these five taxa constrained to monophyly suggests that *P. mariae* may have converged in caudal fin coloration with the other four taxa.

Castro and Vari (2004) recognized that *Prochilodus argenteus, P. costatus, P. harttii* and *P. lineatus* (along with *P. mariae* and the *P. nigricans* group) possess a derived color pattern of wavy lines along the body but were unable to resolve the relationships amongst

them. We similarly found these species to be in close phylogenetic proximity, but were able to completely refine their evolutionary histories. One subclade in our results includes *P. argenteus,* endemic to the Rio São Francisco (but subsequently introduced into several rivers in Northeastern Brazil; Castro and Vari, 2004) and *P. harttii* endemic to the Rio Jequitinhonha and Rio Pardo, coastal rivers of eastern Brazil. Another subclade unites *P. lineatus* from the Río de La Plata system and Rio Paraíba do Sul with *P. costatus* from the Rio São Francisco. Castro and Vari (2004) recognized these as distinct species based on modal differences in scale counts and apparent allopatry, but we find little genetic differentiation, with *P. lineatus* nested within *P. costatus*. It is possible that these entities represent only one species or that they are still in the process of speciation.

The remaining *Prochilodus* species appear broadly within the "*Prochilodus nigricans* group" (Figs. 4 and 5) and several of them may require revision, as their species limits appear varyingly problematic. Clearly, *P. nigricans* as now delimited is not monophyletic and this species will have to be reevaluated, as will the present concept of the polyphyletic *P. rubrotaeniatus*. Two distinct lineages exist within this group, both of which include individuals that fit the current morphological diagnosis of *P. nigricans* or *P. rubrotaeniatus.* For example, two specimens of *P. rubrotaeniatus* from the Corantijn and Marowijne river basins of the Atlantic slope of Suriname nest amongst several specimens of *P. nigricans* from the mainstream of the Rio Amazonas (Iquitos-Peru and Manaus-Brazil). Similarly, two specimens of *P. rubrotaeniatus* from the Essequibo and upper Orinoco river basins, along with *P. lacustris, P. brevis*, and several specimens of *P. nigricans* from eastern Amazonian Brazilian Shield drainages (Rio Araguaia and Rio Tapajós) group in a single clade. Though Turner et al. (2004) also reported a non-monophyletic *P. rubrotaeniatus* (with *P. mariae* nested within it), those authors did not sample *P. brevis, P. lacustris* or *P. nigricans*, and we are not aware of any other suggestions that *P. rubrotaeniatus* or *P. nigricans* may be polyphyletic or paraphyletic.

Clearly a genetic divide exists between major internal clades, but Castro and Vari (2004) did not remark on any morphological differences between *Prochilodus nigricans* from the Rio Araguaia/Rio Tapajós and samples of that species from elsewhere in the Amazon basin. That fact, combined with the presence of specimens that fit the morphological concept of *P. rubrotaeniatus* in both subclades implies that there is no obvious morphological diagnosis for the deepest genetic split within the "*P. nigricans* clade", though geometric approaches may eventually reveal significant differences in mean morphology. The monophyletic components of this larger clade that are morphologically diagnosable (*P. lacustris* and *P. brevis*) are also deeply nested, and their continued recognition as species would imply either recognizing a paraphyletic *P. nigricans* (including a synonymization of *P. rubrotaeniatus* which is taxonomically problematic), or splitting *P. nigricans* into multiple components which may not all be morphologically diagnosable. Further morphological and molecular analyses with extensive sampling from all Amazonian drainages are still necessary to determine which components of the *P. nigricans* complex represent valid species.

Mountain symbols (Figs. 4 and 5) indicate the two clades with trans-Andean distributions. The positions of these clades suggest that the splits separating *Ichthyoelephas* from all other prochilodontids, and *Prochilodus reticulatus* and *P. magdalenae* from most other *Prochilodus*, occurred prior to the final uplift of the Andes approximately 11 million years ago (Wesselingh and Hoorn, 2011). The close relationships between Amazonian and Orinocoan species throughout the phylogeny (Fig. 4) support the hypothesis that Vaupes arch is a barrier between these drainages (Hoorn et al., 1995). However, the presence of *P. rubrotaeniatus* in both basins suggests more recent exchange between the drainages

via dispersal channels, such as the Casiquiare Canal (Winemiller and Willis, 2011). More specific dating and biogeographic inference will require specialized analyses, which will be the focus of a forthcoming paper.

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