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The first molecular phylogeny of Chilodontidae (Teleostei: Ostariophysi: Characiformes) reveals cryptic biodiversity and taxonomic uncertainty

Bruno F. Melo [a,*], Brian L. Sidlauskas [b,c], Kendra Hoekzema [b], Richard P. Vari [c], Claudio Oliveira [a]

[a] Departamento de Morfologia, Instituto de Biociências, UNESP – Universidade Estadual Paulista, Distr. Rubião Jr., 18618-970 Botucatu SP, Brazil
[b] Department of Fisheries and Wildlife, Oregon State University, 104 Nash Hall, 97331-3803, Corvallis OR, USA
[c] Department of Vertebrate Zoology, MCR-159, National Museum of Natural History, Smithsonian Institution, PO Box 37012, 20013-7012, Washington DC, USA

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ABSTRACT

Chilodontidae is a small family of eight described characiform species popularly known as headstanders. These small to moderately sized fishes are well known to aquarists, who prize their striking spotted pigmentation and unusual behaviors, and to systematists, who have revised both chilodontid genera in recent memory and studied their phylogenetic relationships using a comprehensive morphological dataset. However, no molecular phylogeny for the family has ever been proposed. Here, we reconstruct phylogenetic relationships for all eight known chilodontid species using three mitochondrial and two nuclear loci. Results largely agree with the previous morphological hypothesis, and confirm the monophyly of the family as well as its included genera, *Caenotropus* and *Chilodus*. The molecular topology differs slightly from the morphological hypothesis by placing *Caenotropus maculosus* rather than *C. mestomorgmatos* as the sister to the remaining three congeners, and by reconstructing the Curimatidae as the closest outgroup family, rather than the Anostomidae. However, the topologies supported by the morphological data were only slightly less likely and could not be rejected via Shimodaira-Hasegawa tests. Within *Chilodus*, two described species with distinctive pigmentation (*C. fritillus* and *C. zunevei*) appear embedded within the broad distributed *C. punctatus* clade, suggesting the presence of cryptic taxa with polymorphic pigmentation within the present concept of *C. punctatus*. Future work should combine morphological and molecular data to revisit the taxonomy and systematics of *Chilodus* and determine species limits within the *C. punctatus*-group *sensu lato*.

1. Introduction

The 275 species in the fish superfamily Anostomoidea of the order Characiformes (Eschmeyer and Fong, 2013), are widely distributed in Central and South American freshwater ecosystems from Costa Rica to Argentina (Vari, 1983) and in northern South America occur in river systems to both sides of the Andean Cordilleras. Anostomoidea includes the families Anostomidae, Chilodontidae, Curimatidae and Prochilodontidae, that together form a major recognized monophyletic assemblage (Vari, 1983; Buckup, 1998; Sidlauskas, 2008) supported by synapomorphies related to modifications to the gill arches, musculature and dentition. The major clades within the superorder are defined by multiple derived features of those body systems, plus the neurocranium, jaws, connective tissues, pectoral girdle and axial skeleton (Vari, 1983, 1989; Vari et al., 1995; Castro and Vari, 2004; Sidlauskas and Vari, 2008).

Species of Chilodontidae (Fig. 1), one of the four families in the Anostomoidea, adopt a typical head-down orientation while swimming and resting (Vari et al., 1995), an orientation unusual within Characiformes, and are consequently popularly known as headstanders. Given this unusual orientation, small to moderate body sizes and striking pigmentation patterns, chilodontids are well known among aquarists (Isbrücker and Nijssen, 1988). Members of Chilodontidae are broadly distributed in the Amazon and Orinoco river basins, the coastal rivers draining the Guianas, and the Rio Parnaíba basin in northeastern Brazil (Vari and Raredon, 2003; Vari et al., 2009) where they feed on a combination of small invertebrates, sponges and detritus (Goulding et al., 1988; Vari and Raredon, 2003). The family is composed by eight recognized species, six of which were previously studied in taxonomic reviews of *Chilodus* (Isbrücker and Nijssen, 1988) and *Caenotropus* (Vari et al., 1995). Two additional species were described in subsequent decades: *Chilodus fritillus* by Vari and Ortega (1997) and *Caenotropus schizodon* by Scharcansky and Lucena (2007).

Vari (1983) and Vari et al. (1995) proposed a series of synapomorphies for Chilodontidae, as well as for its two included genera, *Caenotropus* and *Chilodus*. In the latter publication, Vari et al.

* Corresponding author.
E-mail address: melo@ibb.unesp.br (B.F. Melo).

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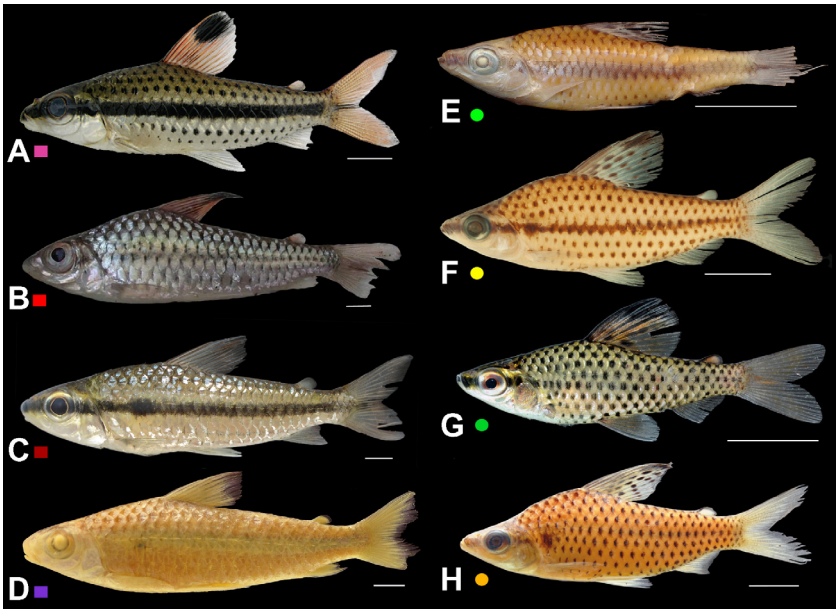

Fig. 1. Representative specimens of the eight known species in Chilodontidae. (A) *Caenotropus maculosus* (live), ANSP 189147, Suriname, Litanie River, Marowijne River basin; (B) *Caenotropus mestomorgmatos* (live), MZUSP 92545, Brazil, Rio Tiquié, tributary of Rio Negro, Amazon basin; (C) *Caenotropus labyrinthicus* (live), MZUSP 95908, Brazil, Rio Teles Pires, tributary of Rio Tapajós, Amazon basin; (D) *Caenotropus schizodon* (preserved), LBP 13847, Brazil, Rio Tapajós, Amazon basin; (E) *Chilodus gracilis* (preserved), LBP 6962, Brazil, Rio Negro, Amazon basin; (F) *Chilodus punctatus* (preserved), AUM 36902, Guyana, Rupununi River, Essequibo River basin; (G) *Chilodus fritillus* (live), AUM 51355, Rio Madre de Diós, a tributary of Mamoré-Madeira system, Amazon basin; (H) *Chilodus zunevei* (preserved), MHNG 2608.040, French Guiana, Kaw River basin. Scale bars indicate one centimeter. Photos by M. Sabaj Pérez (A), F.C.T. Lima (B), J.L.O. Birindelli (C), B.F. Melo (D, E), J. Armbruster (F), N.K. Lujan (G) and B.L. Sidlauskas (H).

(1995) proposed intrageneric phylogenetic relationships for *Caenotropus* on the basis of 10 morphological characters. Scharcansky and Lucena (2007) more recently expanded that analysis to address the phylogenetic placement for *C. schizodon* described in the same publication. No set of phylogenetic relationships within *Chilodus* has ever been proposed. Using a multilocus molecular dataset, Oliveira et al. (2011) corroborated the monophyly of Anostomoidea and of Chilodontidae, though their analysis included only two chilodontid species (*Caenotropus labyrinthicus* and *Chilodus punctatus*). No more detailed molecular hypothesis of phylogenetic relationships within Chilodontidae is available.

Herein, we present the first phylogenetic analysis including all eight species in the Chilodontidae. Our aims were to test the monophyly of Chilodontidae (sensu Vari, 1983) and both genera in the family and to infer their interspecific relationships using a model-based phylogenetic analysis of molecular data. We also discuss the biogeographic distribution of the family, apparent morphological convergences, and the possibility of unrecognized cryptic species within the context of the molecular results.

2. Material and methods

2.1. Taxon sampling

At least one specimen of all eight species of Chilodontidae (Table 1) was included in the analysis. We sampled broadly across the distributional range of *Caenotropus labyrinthicus* and *Chilodus punctatus*, which are the most common species of chilodontids and among the most widespread of the species that have been the subject of recent analysis among all New World characiforms. The map in Fig. 2 illustrates the sampling localities for the ingroup taxa and was prepared using the Quantum GIS 1.7.1and Cartographer module of Mesquite (Maddison and Maddison, 2013). We included several species of each of the other three anostomoid families (Anostomidae, Curimatidae and Prochilodontidae) as outgroups, as well as one species in Hemiodontidae that was used to root the tree (Table 1). Tissues were preserved in 95% ethanol or a saturated DMSO/NaCl solution. Voucher specimens were formalin-fixed, alcohol-preserved and deposited in collections (Table 1; abbreviations follow http://www.asih.org/codons.pdf).

2.2. DNA extraction and sequencing

Total DNA was extracted from ethanol-fixed muscle tissue with a DNeasy Tissue kit (Qiagen Inc.; http://www.qiagen.com) following the instructions of the manufacturer, or following a modified NaCl extraction protocol adapted from Lopera-Barrero et al. (2008). Partial sequences of the genes *16S rRNA* (16S, 608 bp), *cytochrome oxidase C subunit 1* (COI, 633 bp) and *cytochrome B* (Cytb, 985 bp) were amplified using one round of polymerase chain reaction (PCR). Additionally, we obtained sequences of the *myosin heavy chain 6 gene* (Myh6, 704 bp), and *recombination activating gene 1* (Rag1, 1210 bp) through nested-PCR following the procedures detailed in Oliveira et al. (2011). PCR amplifications were performed in 12.5 μl reactions containing 9.075 μl of double-distilled water, 1.25 μl 5 × reaction buffer, 0.375 μl $MgCl_2$, 0.25 μl dNTP mix at 8 mM, 0.25 μl of each primer at 10 μM (list of primers in Table 2), 0.05 μl Platinum Taq DNA polymerase enzyme (Invitrogen; www.invitrogen.com) and 1.0 μl genomic DNA (10–50 ng). The amplification cycles consisted of an initial denaturation (4 min at 95 °C) followed by 28 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). All PCR products were visually identified in a 1% agarose gel. Samples were cleaned using ExoSAP (Hanke and Wink, 1994) and subsequently sequenced using dye terminators (Big Dye™ Terminator v 3.1 Cycle Sequencing Ready Reaction Kit, Applied Biosystems; http://www.appliedbiosystems.com) purified again through ethanol precipitation and loaded onto an automatic sequencer ABI 3130-Genetic Analyzer (Applied Biosystems) at either the Universidade Estadual Paulista, Botucatu, São Paulo, Brazil, or Oregon State University, Corvallis, Oregon, USA. Consensus sequences were assembled and edited in BioEdit 7.0.9.0 (Hall,

Table 1
Genus and species, voucher specimens, locality information and GenBank accession number of chilodontids and outgroup taxa used in this study. Asterisks show sequences obtained by Oliveira et al. (2011).

Species	Voucher	Specimen	Locality	Coordinates	City, State	Country	16s	COI	Cytb	Myh6	Rag1
Caenotropus labyrinthicus	LBP 1828	12912	Rio Araguaia, Amazon basin	15°53′35.6″S/52°15′01″W	Aragarças, Goiás	Brazil	–	EU185613*	HQ289538*	–	HQ289154*
Caenotropus labyrinthicus	LBP 9216	43161	Río Apure, Orinoco basin	07°37′24.4″N/66°24′48″W	Cabruta, Guárico	Venezuela	HQ171428*	–	–	HQ289136*	HQ289327*
Caenotropus labyrinthicus	LBP 3050	19138	Río Orinoco, Orinoco basin	07°38′11.6″N/66°19′4.2″W	Caicara del Orinoco, Bolívar	Venezuela	KF562379	–	KF562437	KF562462	KF562485
Caenotropus labyrinthicus	OS 18770	PE10-82	Río Nanay, Amazon basin	3.751667S/73.287222 W	Iquitos, Loreto	Peru	KF562380	KF562408	KF562438	KF562463	KF562486
Caenotropus maculosus	MHNG 2705.038	157-13	Sipaliwini, Corantijn basin	04°38′48.3″N/57°12′53″W	Sipaliwini	Suriname	KF562381	KF562409	KF562439	KF562464	KF562487
Caenotropus maculosus	MHNG 2717.052	157-15	Tapanahony river, Marowijne basin	04°15′0″N/54°31′33.2″W	Sipaliwini	Suriname	KF562382	KF562410	KF562440	KF562465	KF562488
Caenotropus maculosus	ANSP 189156	6895	Marowijne river basin	3°17′24″N/54° 4′38″W 54°4′38′W	Sipaliwini	Suriname	KF562383	KF562411	KF562441	–	KF562489
Caenotropus mestomorgmatos	ANSP 180516	PEL02-T48	Río Nanay, Amazon basin	3°46′45″S/73°22′6″W	Iquitos, Loreto	Peru	KF562384	KF562412	KF562442	KF562466	KF562490
Caenotropus mestomorgmatos	OS 18346	PE10-67	Río Nanay, Amazon basin	3.751667S/73.31625W	Iquitos, Loreto	Peru	KF562385	KF562413	KF562443	KF562467	KF562491
Caenotropus mestomorgmatos	OS 18772	PE10-93	Río Nanay, Amazon basin	3.751667S/73.287222W	Iquitos, Loreto	Peru	KF562386	KF562414	KF562444	KF562468	KF562492
Caenotropus mestomorgmatos	OS 18323	PE10-139	Río Nanay, Amazon basin	3.780972S/73.363889W	Iquitos, Loreto	Peru	KF562387	KF562415	KF562445	–	–
Caenotropus mestomorgmatos	OS 18323	PE10-140	Río Nanay, Amazon basin	3.780972S/73.363889W	Iquitos, Loreto	Peru	KF562388	KF562416	KF562446	–	–
Caenotropus schizodon	LBP 13847	57304	Rio Tapajós, Amazon basin	04°16′49″S/59°59′26.1″W	Itaituba, Pará	Brazil	KF562389	–	KF562447	–	–
Caenotropus schizodon	LBP 13847	57305	Rio Tapajós, Amazon basin	04°16′49″S/59°59′26.1″W	Itaituba, Pará	Brazil	KF562390	KF562417	KF562448	–	KF562494
Chilodus fritillus	AUM 51355	T10200	Río Madre de Diós, Mamoré-Madeira system, Amazon basin	12.27713S/69.15237W	Madre de Dios	Peru	KF562391	KF562418	–	–	KF562495
Chilodus gracilis	LBP 6962	33397	Rio Negro, Amazon basin	00°00′32.1″N/66°55′35.7″W	São Gabriel da Cachoeira, Amazonas	Brazil	KF562392	KF562419	KF562449	KF562470	KF562496
Chilodus gracilis	LBP 6962	33398	Rio Negro, Amazon basin	00°00′32.1″N/66°55′35.7″W	São Gabriel da Cachoeira, Amazonas	Brazil	KF562393	KF562420	KF562450	KF562471	KF562497
Chilodus gracilis	LBP 7026	34094	Rio Negro, Amazon basin	00°16′25.9″N/66°38′36.5″W	São Gabriel da Cachoeira, Amazonas	Brazil	KF562394	KF562421	KF562451	KF562472	KF562498
Chilodus gracilis	LBP 7026	34095	Rio Negro, Amazon basin	00°16′25.9″N/66°38′36.5″W	São Gabriel da Cachoeira, Amazonas	Brazil	KF562395	KF562422	KF562452	KF562473	KF562499
Chilodus punctatus	LBP 11921	62056	Rio Purus, Amazon basin	10°04′44.3″S/67°32′33.9″W	Rio Branco, Acre	Brazil	KF562396	KF562423	KF562453	KF562474	KF562500
Chilodus punctatus	LBP 11921	62057	Rio Purus, Amazon basin	10°04′44.3″S/67°32′33.9″W	Rio Branco, Acre	Brazil	KF585008	KF562424	KF585014	KF585017	–
Chilodus punctatus	LBP 4090	23527	Rio Juruá, Amazon basin	07°34′28.8″S/72°55′24.9″W	Mâncio Lima, Acre	Brazil	HQ171309*	–	HQ289598*	–	HQ289211*
Chilodus punctatus	LBP 12041	51554	Rio Purus, Amazon basin	07°56′11.0″S/63°27′35.3″W	Lábrea, Amazonas	Brazil	KF562398	KF562425	KF562455	KF562475	KF562502
Chilodus punctatus	LBP 9391	42598	Rio Guamá, Amazon basin	01°34′00″S″/47°09′51.4″W	Ourém, Pará	Brazil	KF562399	KF585011	KF562456	KF562476	KF562503
Chilodus punctatus	LBP 7202	34864	Rio Araguaia, Amazon basin	15°32′25.8″S/52°26′18.7″W	Barra do Garças, Mato Grosso	Brazil	KF562400	KF562426	KF562457	KF562477	KF562504
Chilodus punctatus	ANSP 180521	PEL03-T63	Rio Nanay, Amazon basin	3°52′21″S/73° 32′43″W	Iquitos, Loreto	Peru	KF562401	KF562427	–	–	KF562505
Chilodus punctatus	OS 18781	PE10-83	Rio Nanay, Amazon basin	3.751667S/73.287222 W	Iquitos, Loreto	Peru	KF562402	KF562428	–	KF562479	KF562506
Chilodus punctatus	OS 18781	PE10-100	Rio Nanay, Amazon basin	3.751667S/73.287222 W	Iquitos, Loreto	Peru	KF562403	KF562429	KF562458	–	KF562507
Chilodus punctatus	OS 18318	PE10-143	Rio Nanay, Amazon basin	3.780972S/73.363889 W	Iquitos, Loreto	Peru	KF562404	KF562430	–	–	–
Chilodus punctatus	LBP 15541	61601	Rio Takutu, Rio Branco, Amazon basin	03°22′55.9″N/59°51′28.3″W	Bonfim, Roraima	Brazil	KF562405	–	KF562459	KF562482	KF562508
Chilodus punctatus	LBP 15541	61602	Rio Takutu, Rio Branco, Amazon basin	03°22′55.9″N/59°51′28.3″W	Bonfim, Roraima	Brazil	KF562406	KF562431	KF562460	KF562483	KF562509
Chilodus zunevei	MHNG 2705.043	157-14	Commewijne river basin	05°23′47.50”N/ 54°44′9.17”W	Para	Suriname	KF585009	KF585012	KF585015	KF585018	KF585020
Anostomus ternetzi	LBP 4375	24146	Rio Branco, Amazon basin	02°18′02.0″N/60°55′20.7″W	Mucajaí, Roraima	Brazil	HQ171317*	–	HQ289606*	HQ289026*	HQ289219*
Anostomus ternetzi	MZUSP 97271	7163	Rio Tapajós, Amazon basin	08° 11′4.0″S/55°10′47.0″W	Novo Progresso, Pará	Brazil	KF585010	KF585013	KF585016	KF585019	KF585021
Leporinus altipinnis	LBP 4459	24381	Rio Negro, Amazon basin	00°40′03.1″S/62°58′23.5″W	Barcelos, Amazonas	Brazil	HQ171321*	–	HQ289610*	HQ289030*	HQ289223*
Schizodon scotorhabdotus	LBP 3046	19130	Río Orinoco, Orinoco basin	07°38′11.6″N/66°19′04.2″W	Caicara del Orinoco, Bolívar	Venezuela	HQ171270*	KF562432	HQ289559*	HQ288980*	HQ289177*

Table 1 (*continued*)

Species	Voucher	Specimen	Locality	Coordinates	City, State	Country	16s	COI	Cytb	Myh6	Rag1
Schizodon vittatus	LBP 3994	23098	Rio Araguaia, Amazon baisn	11°40′9″S/50°51′0.30″W	São Félix do Araguaia, Mato Grosso	Brazil	HQ171308*	–	HQ289597*	HQ289018*	HQ289210*
Curimatella dorsalis	LBP 3759	22034	Rio Paraguay, La Plata basin	19°34′33.7″S/56°14′49.5″W	Aquidauana, Mato Grosso do Sul	Brazil	HQ171290*	KF562433	HQ289579*	HQ289000*	HQ289194*
Cyphocharax aspilos	LBP 6109	29560	Lago Maracaibo	09°38′53.8″N/72°34′56.4″W	Machiques de Perijá, Zulia	Venezuela	HQ171363*	–	HQ289650*	HQ289071*	HQ289264*
Cyphocharax gouldingi	LBP 1537	11889	Rio Araguaia, Amazon basin	15°53′53.4″S/52°13′00.6″W	Aragarças, Goiás	Brazil	HQ171243*	KF562434	HQ289534*	HQ288953*	HQ289150*
Potamorhina altamazonica	LBP 2571	17020	Rio Purus, Amazon basin	08°51′21.5″S/68°42′22.6″W	Boca do Acre, Amazonas	Brazil	HQ171261*	–	HQ289552*	HQ288971*	HQ289168*
Steindachnerina brevipinna	LBP 5185	26336	Rio Paraná, La Plata basin	22°47′29″S/53°20′58″W	Porto Rico, Paraná	Brazil	HQ171339*	–	HQ289628*	HQ289048*	HQ289241*
Hemiodus immaculatus	LBP 1725	12849	Rio Negro, Amazon basin	02°03′10.0″S/60°06′31.7″W	Manaus, Amazonas	Brazil	HQ171246*	–	HQ289537*	HQ288956*	HQ289153*
Prochilodus reticulatus	LBP 6127	29514	Rio Catacumbo, Lago Maracaibo	09°05′08.3″N/72°13′50.5″W	Catacumbo, Zulia	Venezuela	HQ171358*	KF562435	HQ289647*	HQ289067*	HQ289260*
Semaprochilodus laticeps	LBP 1383	12728	Río Orinoco, Orinoco basin	02°03′10.0″S/60°06′31.7″W	Caicara del Orinoco, Bolívar	Venezuela	HQ171245*	KF562436	HQ289536*	HQ288955*	HQ289152*

1999) and Geneious 6.1 (Biomatters, 2013). Where uncertainty of nucleotide identity was detected, IUPAC ambiguity codes were applied.

2.3. Alignment and phylogenetic analyses

Consensus sequences of each gene for each individual were independently aligned using the Muscle program (Edgar, 2004) under default parameters. The resulting alignments were inspected by eye for obvious misalignments that were then corrected manually. GenBank accession numbers appear in Table 1 and the matrix was deposited in TreeBase (http://treebase.org) under number 14605. To evaluate the occurrence of substitution saturation, the index of substitution saturation (Iss) as described by Xia et al. (2003) and Xia and Lemey (2009) was estimated using Dambe 5.3.38 (Xia, 2013). The nucleotide frequencies were computed in MEGA 5.0 (Tamura et al., 2011).

Maximum likelihood (ML) analyses were generated in a partitioned (13 partitions, Table 3) RAxML (Stamatakis, 2006) analysis using the CIPRES web server (Miller et al., 2010). Random starting trees were used for ML tree search and all other parameters were set to default values. All ML analyses were performed under GTR + G since RAxML only applies this model (Stamatakis et al., 2008). The robustness of the topology was investigated using 1000 bootstrap pseudoreplicates.

Maximum parsimony (MP) analysis was performed using PAUP* 4.0b10 (Swofford, 2003). Heuristic searches were performed with minimally 1000 random addition replicates and TBR branch swapping. All characters were unordered and all transformation series were equally weighted. Branches with maximum length of zero were collapsed. Gaps were treated as missing data. The resulting topologies were statistically tested with the bootstrap method (Felsenstein, 1985) using 1000 pseudoreplicates.

We inferred a Bayesian topology (BI) with a partitioned matrix using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) via the CIPRES web portal (Miller et al., 2010). Schema ranging from 1 to 13 partitions was tested following Li et al. (2007) under the Akaike information criterion (AIC) as detailed in Table 3 and in Supplementary data 1. Parameters were estimated using ModelTest 3.6 (see Posada and Crandall, 1998 for model symbols) implemented in PAUP* 4.0b10 (Swofford, 2003) on each partition (Table 3). We performed two runs of four independent MCMC chains with 10 million replicates each, sampling one tree every one thousand generations. The distributions of log likelihood scores were examined using Tracer 1.5 (Rambaut and Drummond, 2007) in order to determine stationarity and decide if extra runs were required to achieve convergence. The first one million generations (10%) were discarded as burn-in, and the remaining trees were used to construct a 50% majority rule consensus tree in PAUP* (Swofford, 2003).

To test the degree of support for the resulting molecular phylogenies versus the previously published morphological hypothesis, we compared the maximum likelihood unconstrained tree to the maximum likelihood trees generated under two different constraint trees in RAxML. In the first, we constrained the four anostomoid families to conform to the arrangement proposed by Vari (1983) in which Anostomidae and Chilodontidae are sister taxa, as are Curimatidae and Prochilodontidae. In the second, the ingroup Chilodontidae was constrained to conform to the morphological hypothesis of relationships within *Caenotropus* of Vari et al. (1995), modified by the addition of *C. schizodon* as sister to *C. labyrinthicus* as proposed by Scharcansky and Lucena (2007). Constraint trees were constructed in Mesquite (Maddison and Maddison, 2013). We compared the maximum likelihood topologies inferred under these three scenarios using the Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999) as implemented

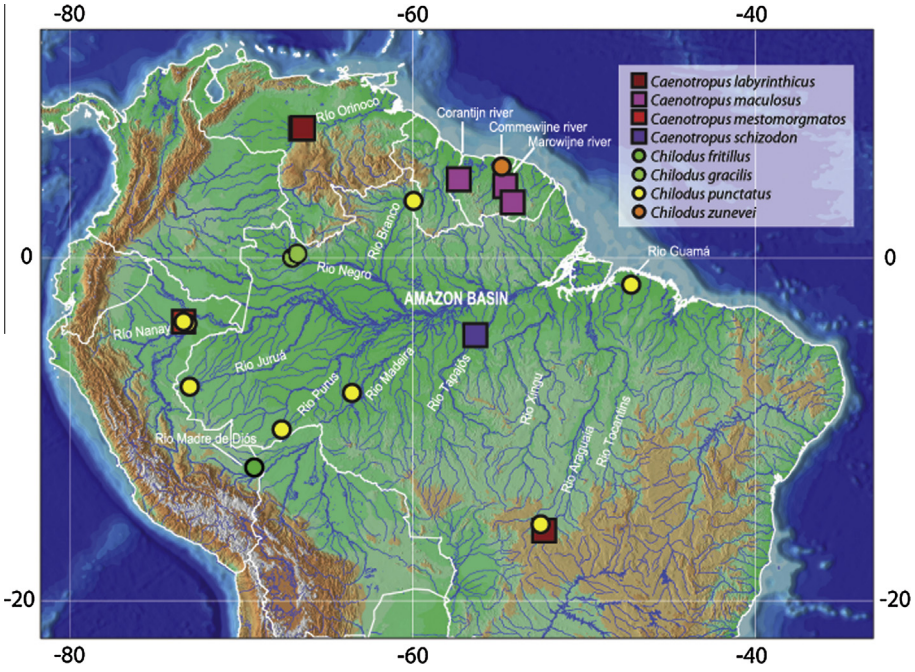

Fig. 2. Distribution map of sampled specimens of Chilodontidae. Outgroups are not shown.

Table 2
Information content and characteristics of each gene.

Gene	Bp after alignment	PCR	Primer sequence (5′–3′)	Reference	Π_A	Π_C	Π_G	Π_T
16S	608	1 PCR	16Sa-L – ACGCCTGTTTATCAAAAACAT 16Sb-H – CCGGTCTGAACTCAGATCACGT	Palumbi (1996)	0.308	0.242	0.221	0.228
COI	633	1 PCR	L6252-Asn – AAGCCGGGGAAAGCCCCGGCAG H7271-COXI – TCCTATGTAGCCGAATGGTTCTTTT	Melo et al. (2011)	0.245	0.258	0.181	0.314
Cytb	985	1 PCR	LNF – GACTTGAAAAACCAYCGTTGT H08R2 – GCTTTGGGAGTTAGDGGTGGGAGTTAGAATC	Oliveira et al. (2011)	0.276	0.297	0.140	0.285
Myh6	704	1st PCR	F329 – CCGCMTGGATGATCTACAC A3R1 – ATTCTCACCACCATCCAGTTGAA	Li et al. (2007)	0.306	0.221	0.249	0.241
		2nd PCR	A3F2 – GGAGAATCARTCKGTGCTCATCA A3R2 – CTCACCACCATCCAGTTGAACAT					
Rag1	1210	1st PCR	Rag1CF1 – ACCCTCCGTACTGCTGAGAA Rag1CR1 – CGTCGGAAGAGCTTGTTGCC	Oliveira et al. (2011)	0.257	0.238	0.281	0.222
		2nd PCR	Rag1CF2 – TACCGCTGAGAAGGAGCTTC Rag1CR2 – TGTTGCCAGACTCATTGCCCTC					

Table 3
Gene partitions and their models as selected by ModelTest.

Gene and position	Partitions	Best-fit model
16S	1–608	GTR + G + I
COI 1st position	609–1241/3	TrN + G
COI 2nd position	610–1241/3	F81
COI 3rd position	611–1241/3	GTR + G
Cytb 1st position	1242–2226/3	TrN + I
Cytb 2nd position	1243–2226/3	SYM + I + G
Cytb 3rd position	1244–2226/3	TrN + I
Myh6 1st position	2227–2930/3	GTR + G + I
Myh6 2nd position	2228–2930/3	GTR + I
Myh6 3rd position	2229–2930/3	HKY + G
Rag1 1st position	2931–4140/3	TVM + I + G
Rag1 2nd position	2932–4140/3	TVM + G
Rag1 3rd position	2933–4140/3	TVM + I + G

in the phangorn (Schliep, 2011) package in R (R Development Core Team, 2013). 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 computed likelihoods and *p*-values with and without optimizing the rate matrix and base frequencies in phangorn (Table 4, Supplementary figures 1 and 2). We also filtered the combined results from both Bayesian Markov chains to determine the percentage of trees in the posterior distribution that were consistent with bipartitions present in the morphological reconstruction, but not present in the best-supported molecular reconstruction.

3. Results

Most sequences from the outgroup species were previously published in the phylogeny of the Characidae by Oliveira et al. (2011); however, the sequences from the *cytochrome oxidase C subunit I* (COI) for these species were newly generated for this study. The concatenated matrix from 13 outgroups and 32 specimens of the Chilodontidae include 4140 bp and 1506 variable sites of which 1179 were parsimony informative. The Iss index indicated no saturation in transitions or transversions in both asymmetrical (Iss.cAsym) and symmetrical (Iss.cSym) topologies. Table 2 contains numbers of base pairs (bp) after alignment, primer sequences, and nucleotide composition for each analyzed gene. Comparisons of log likelihoods, AIC and BIC values among different partitioning

schemes (from 1 to 13 partitions) were tested and are presented in Supplementary data 1.

Throughout the text and in Fig. 3, measures of support are indicated as a series of three numbers on selected internal branches of the trees subtending labeled clades, starting with posterior probabilities in Bayesian Inference (BI) analysis and followed by non-parametric bootstrap percentages from maximum likelihood (ML) and parsimony (MP) analyses, respectively (e.g., 1/100/100, see Fig. 3); dashes represent values lower than 0.9 (BI) or 50% (ML and MP) and asterisks represent nodes that have different topologies in different analytical methods. Nodes without support values greater than 0.9 (BI) and 50% (ML and MP) were collapsed.

The Bayesian results represent a majority rule consensus of 18002 post-burn-in trees, the likelihood analysis yielded a single tree with a sum of branch lengths (SBL) of 1.931, and the parsimony analysis returned a single tree (TL: 4726; CI: 0.439; RI: 0.714). Fig. 3 shows the maximum likelihood topology, along with bootstrap and posterior probabilities values from the three analyses, all of which returned very similar results. Anostomidae, Chilodontidae, Curimatidae and Prochilodontidae were all corroborated as monophyletic by all three analyses with 100% bootstrap support or posterior probability equal to one. We obtained a well-supported clade (1/93/70) composed of the Chilodontidae, Curimatidae and Prochilodontidae. The Bayesian consensus differs from the maximum likelihood topology only in a single relationship among the species of the outgroup Curimatidae. The clade composed by Chilodontidae and Curimatidae was well-supported in BI (0.9) and ML (82) but with low support in the MP reconstruction (41). Otherwise, optimal topologies from the three analyses agree completely.

Within Chilodontidae (clade 1), the monophyly of *Caenotropus* (clade 2) was also well-supported with *C. maculosus* (clade 3) as the sister of a clade composed by its three congeners. In clade 4, the analysis placed *C. mestomorgmatos* as sister to clade 5 composed of *C. labyrinthicus* and *C. schizodon*. Clade 6 is composed by two specimens of *C. schizodon* from the Rio Tapajós in the eastern portions of the Amazon basin and one specimen of *C. labyrinthicus* from the Río Nanay in Peru (Fig. 2), in the western portion of that river system, thereby rendering *C. labyrinthicus* paraphyletic (albeit with only moderate statistical support: -/71/63).

Our results corroborated *Chilodus* as a monophyletic genus (clade 7). Within *Chilodus*, *C. gracilis* (from the Rio Negro in Brazil) appeared as monophyletic (clade 8) and as sister to clade 9 containing the other three currently recognized species of the genus (*C. fritillus, C. punctatus* and *C. zunevei*). *Chilodus punctatus*, however, was not recovered as a monophyletic group as a consequence of *C. fritillus* and *C. zunevei* nesting among the twelve sampled individuals of *C. punctatus*.

Within clade 9, two lineages were recovered. The first is composed solely by *Chilodus punctatus* from the Rio Araguaia, a large river south of the mainstream Amazon that drains a portion of the Brazilian Shield and flows into the Rio Tocantins and through that river to the lower Rio Amazonas (Fig. 2). The second lineage (clade 9, 0.8/92/68) is composed by specimens of *Chilodus punctatus* from the remainder of the Amazon, including the Rios Juruá, Purus and Nanay in the west, the Rio Takutu at the border of Brazil and Guyana (a tributary of the upper Rio Branco) in the northeast, and the Rio Guamá (a southern tributary of the lower Amazon) in the east (Fig. 2). Clade nine also includes *C. fritillus* from south-eastern Peru in the Río Madre de Diós, an upper tributary to the Rio Madeira in the western Amazon and a specimen of *C. zunevei* from the Atlantic slope of the Guianas (Commewijne River, Suriname) in north-eastern South America.

While the tree topology discussed above is that best supported by the available molecular data, the maximum likelihood trees under the topological constraints based on previous morphological results (Supplementary figures 1 and 2) are only slightly less likely (Table 4). Shimodaira-Hasegawa tests failed to reject these two alternative topologies (Table 4). Nevertheless, no sampled trees in the Bayesian posterior distributions are fully congruent with the morphological topologies.

4. Discussion

4.1. Interfamilial relationships within Anostomoidea

Our molecular study returns the same hypothesis of close relationship among Chilodontidae, Curimatidae and Prochilodontidae obtained by Oliveira et al. (2011). That congruence is perhaps unsurprising given the similarity of loci and taxa examined in the two studies. This arrangement differs from the interfamilial hypothesis ((Anostomidae + Chilodontidae) + (Curimatidae + Prochilodontidae)) of Vari (1983, 1989), which was based on synapomorphies of multiple morphological systems. The latter result was subsequently obtained by Buckup (1998) using much of the same data. The arrangement of families suggested by the molecular data would imply a large number of morphological convergences or reversals and indicate a much more complex evolutionary history of these fishes than previously suspected. Of particular note would be the very distinctive shared modifications of the gill arches in the Chilodontidae and Anostomidae, which include pronounced enlargement of the upper and lower pharyngeal dentition, the presence of two or more pointed cusps on those teeth, a shift in alignment of the fourth upper pharyngeal tooth plate, vertical expansion of the fifth upper pharyngeal tooth plate, a highly developed *obliquees dorsalis* muscle on the fourth infrapharyngobranchial and cord-like ligaments joining the ectopterygoid and ventral wing of the lateral ethmoid; none of which occur elsewhere in Characiformes (Vari, 1983). Under the scenario implied by the molecular data, either all of these characters are convergent, or they were present in the common ancestor of Anostomoidea and subsequently lost during the evolution of Curimatidae and Prochilodontidae.

The strength of the morphological data makes it noteworthy that the currently available genetic data do not strongly reject the morphological hypothesis of interfamilial relationships. Support for the novel arrangement based on molecular evidence was inconsistent among the three methods that we employed, and support for the maximum likelihood tree that conforms to the morphological hypothesis (Supplementary figure 1) was similar enough to the support for the unconstrained tree that a Shimoda-

Table 4
Results of Shimodaira-Hasegawa tests of alternative topologies with and without optimization of base frequencies and rate matrices in Phangorn. *P*-values lower than 0.05 would indicate statistical rejection of equivalence of the topologies.

Constraint	No optimization			Optimized		
	lnL	∂L	*p*-Value	lnL	∂L	*p*-Value
None	−13134.40	0.00	0.7224	−12047.94	0.00	0.7317
Caenotropus (Vari et al., 1995)	−13136.61	2.21	0.4502	−12048.90	0.97	0.5345
Outgroups (Vari, 1983)	−13141.15	6.76	0.1506	−12050.72	2.78	0.3413

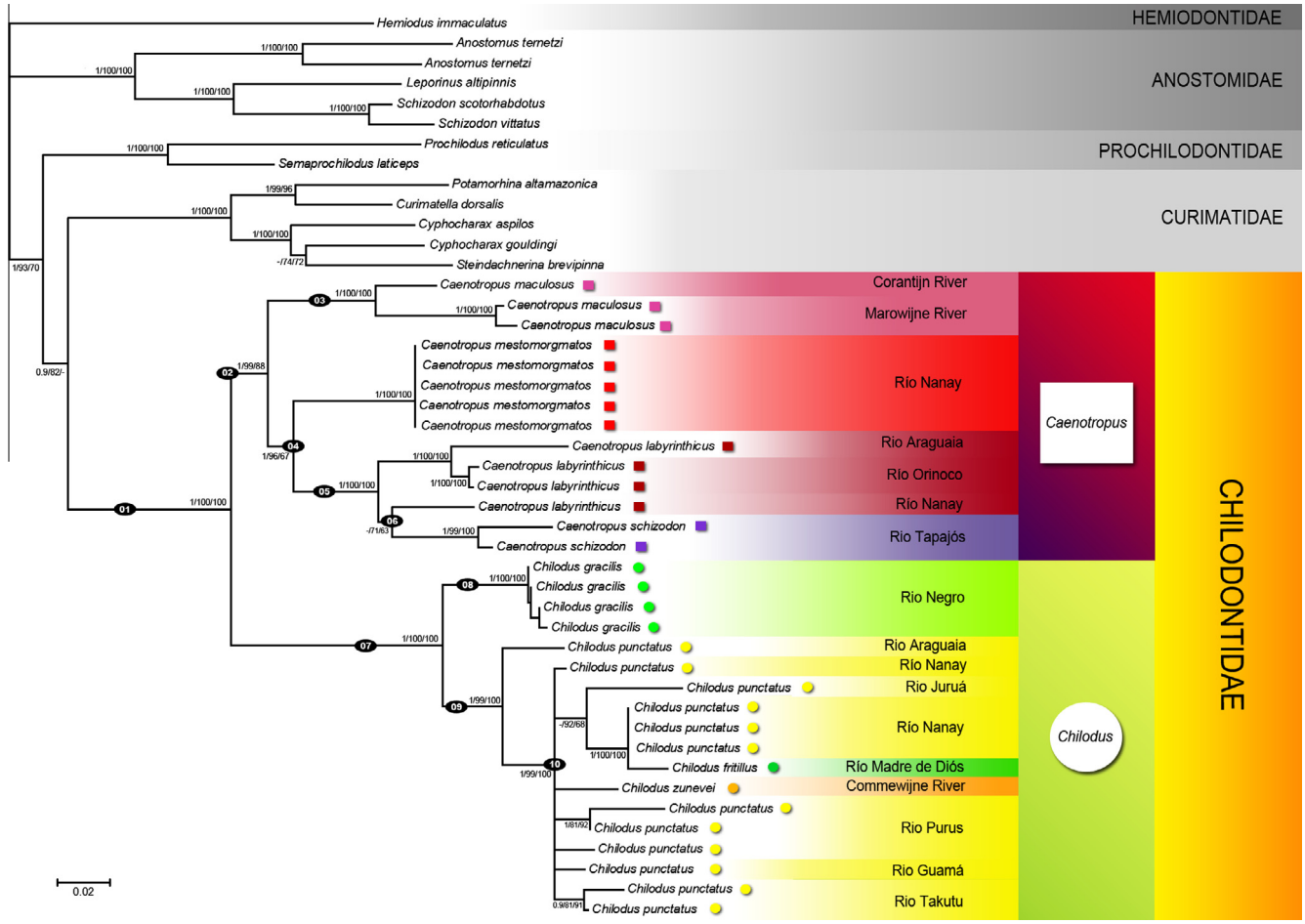

Fig. 3. Relationships among species of Chilodontidae and others taxa of the Anostomoidea obtained by a ML partitioned analyses of the concatenated dataset. A series of three numbers (e.g. 1/100/100) at each of the main nodes shows the posterior probability for that split obtained in BI, percentage of bootstrap support obtained by ML, and percentage of bootstrap obtained by MP analysis, respectively (1000 bootstrap pseudoreplicates). Dashes show values lower than 0.9 (BI) or 50% (ML, MP) and nodes not supported by values higher than 0.9 (BI) or 50% (ML, MP) were collapsed. Asterisks represent nodes that were not obtained by BI or MP analyses. Clades with dark numbered ovals are discussed in the text.

ira-Hasegawa test was unable to distinguish between these hypotheses (Table 4). None of the sampled trees in the Bayesian posterior conform exactly to the morphological hypothesis of relationships among these families, but substantial variation in results exist. The posterior distribution includes a moderate percentage (4.44%) of topologies in which Chilodontidae appears as sister to Prochilodontidae as well as more limited occurrence (<1%) of sister relationships between Anostomidae and Curimatidae, Anostomidae and Prochilodontidae, and Curimatidae and Prochilodontidae. We plan to examine these alternatives more thoroughly in an upcoming analysis with more complete taxon and gene sampling. Such further tests are outside the scope of the present paper, which focuses on Chilodontidae.

4.2. Monophyly of Chilodontidae and included genera

Our results support the monophyly of the family Chilodontidae, corroborating the previous morphological hypothesis of Vari (1983) and Vari et al. (1995) and agreeing with Greenwood et al. (1966), who in a pre-cladistic analysis were the first authors to elevate chilodontids to the familial level. Subsequently, Vari (1983) proposed the monophyly of the family Chilodontidae based on 26 synapomorphies (his characters 74–99) mostly related to the gill-arches, suspensorium, pectoral girdle, and anterior portions of the vertebral column. In a taxonomic revision of *Caenotropus*, Vari et al. (1995) discovered 10 additional synapomorphies (their characters 27–36) for the family involving a variety of osteological and scale-based characters. Oliveira et al. (2011) later obtained monophyly of the two species of Chilodontidae included in their molecular study.

Clade 2, containing the four species of *Caenotropus*, corroborates the monophyly of that genus as hypothesized by Vari et al. (1995) on the basis of a series of eight synapomorphies involving details of the bones of the infraorbital series, jaws, neurocranium and scales among others. Similarly, clade 7, comprising all species and multiple specimens of *Chilodus* from different localities, appears as a strongly supported monophyletic lineage (1/100/100). Vari et al. (1995) has previously proposed monophyly of *Chilodus* based on eight synapomorphies related to modifications of the bones of the infraorbital series, the lower jaw, the hyoid series, the pattern of the laterosensory canals in the dentary, infraorbitals and neurocranium and the complete loss of the third postcleithrum.

4.3. Interspecific relationships in Caenotropus

Vari et al. (1995) distinguished *Caenotropus mestomorgmatos* from its congeners as a new species on the basis of the combination of various features. Two of these, the presence of three scales (versus four) above the lateral line to the dorsal fin and a broad region of dusky pigmentation across most of the dorsal fin (versus no pigmentation or just a distal spot of pigmentation) were unique to the species in the genus and with the latter proposed as an autapomorphy for

the species. Those authors proposed that *C. mestomorgmatos* was, in turn, the sister species to a clade composed of *C. maculosus* and *C. labyrinthicus* and supported their proposal with three synapomorphies involving different portions of the body. Scharcansky and Lucena (2007) subsequently described *C. schizodon* and hypothesized a sister relationship between that new species and *C. labyrinthicus*.

Our results are incongruent with those hypotheses, and reverse the position of *C. maculosus* and *C. mestomorgmatos* proposed by Vari et al. (1995). The results of this study consequently place *C. maculosus* as sister to the group containing the remaining three species in the genus (*C. labyrinthicus, C. mestomorgmatos* and *C. schizodon*) with *C. mestomorgmatos* as sister to a clade formed by *C. labyrinthicus* and *C. schizodon* (Fig. 3). The arrangement supported by the morphological analysis is only slightly less likely than that based on the molecular data, and the two topologies could not be distinguished by the Shimodaira-Hasegawa test (Table 4). However, no trees in the Bayesian posterior place *C. mestomorgmatos* as sister to its remaining congeners, and the constrained maximum likelihood tree (Supplementary figure 2) includes a very short internode that effectively creates a polytomy among *C. mestomorgmatos*, *C. maculosus* and a clade containing *C. labyrinthicus* and *C. schizodon*. Thus, the molecular support for the novel hypothesis of relationships within *Caenotropus* is relatively robust, and it is worth exploring whether morphological support for the basal position of *C. maculosus* exists.

As pointed out by Vari et al. (1995), all species of *Chilodus* have darkly pigmented distal portions of the dorsal fin (Fig. 1, see also Isbrücker and Nijssen, 1988). Under our molecular reconstruction, *Caenotropus maculosus* shares this plesiomorphic condition, as does *C. mestomorgmatos* to a fainter extent. Their remaining congeners would then possess a synapomorphic reduction in dorsal-fin pigmentation (Fig. 1).

Our results also suggest the possibility of cryptic diversity within the present concept of *Caenotropus maculosus.* A deep split occurs within Clade 3, separating a specimen from the Corantijn River basin of western Suriname from two others that originated in the Marowijne River system of eastern Suriname and western French Guiana. Within the intervening drainages, *C. maculosus* is only known from the Suriname River which lies close to the Marowijne basin (Sidlauskas and Vari, 2012; Mol et al., 2012). The evidence suggests the presence of two genetically isolated populations. Additional studies should investigate whether these merit species status.

Our results corroborate the close relationship between *Caenotropus labyrinthicus* and *C. schizodon* (clade 5) as proposed by Scharcansky and Lucena (2007). These species share the three character states originally discovered by Vari et al. (1995) as autapomorphies for *C. labyrinthicus* and later proposed as synapomorphies for that species and *C. schizodon* by Scharcansky and Lucena (2007). *Caenotropus labyrinthicus* resolved as paraphyletic in our analyses (clade 5) due to a single individual of that species from the western Amazon clustering with two individuals of *C. schizodon* of the eastern Amazon (clade 6). This relationship received only moderate statistical support (–/71/63), and a substantial fraction (22.4%) of the trees in the posterior distribution include a monophyletic *C. labyrinthicus*. The apparent paraphyly of that species in the best-supported tree may represent an artifact of locus selection or model choice, or gene-tree conflict resulting from incomplete lineage sorting. Alternatively, it could reflect the presence of multiple species within the present concept of *C. labyrinthicus*, or indicate that *C. labyrinthicus* and *C. schizodon* are morphotypes of the same species. The samples of *C. schizodon* used herein originated in the Rio Tapajós, a drainage which includes the type locality of that species. However, several individuals of *C. labyrinthicus* with bifid premaxillary teeth, the purported distinguishing feature of *C. schizodon,* have been found in other portions of the Amazon basin including the Rio Madeira of the western Amazon (J. Zuanon, pers. com.) and Rio Xingu of the Brazilian Shield (observed in this study). Studies of other populations of *C. labyrinthicus* and *C. schizodon* across the range of those species within the Amazon and Tocantins basins are required to evaluate the distribution and intrapopulational consistency of the dentition characters that putatively separate these species, and to further evaluate their reciprocal monophyly.

As a final unanswered question within *Caenotropus*, we note that the known distribution of *Caenotropus mestomorgmatos* is pronouncedly disjunct. One population is distributed in blackwater portions of the southern Río Orinoco of Venezuela and the adjoining upper reaches of the Rio Negro of Brazil (Vari et al., 1995), and another in the blackwater Río Nanay of northeastern Peru (Vari and Ortega, 1997). All of our sequenced samples are from the Peruvian population. In a future study, it would be of great interest to determine whether these two populations have diverged enough genetically to merit possible recognition as separate species.

4.4. Phylogenetic relationships and comments on the taxonomy of *Chilodus*

Chilodus is a well known genus among aquarists and includes four currently valid species (Vari and Ortega, 1997) and we herein present the first phylogenetic reconstruction for the genus. Within the monophyletic *Chilodus*, *C. gracilis* appears as the sister group of a major clade (9) including the remaining three species. Relationships within clade 9 indicate that the present concept of *C. punctatus* is paraphyletic unless *C. fritillus* and *C. zunevei* are included within *C. punctatus.* Within this broadly defined *C. punctatus* species-complex (clade 9), we found two distinct genetic lineages. The first is restricted to the Rio Araguaia, a lowland river draining into the Rio Tocantins system to the southeast of the Amazon basin and which represents an ecoregion with one of the highest degrees of ichthyological endemism in the Neotropics (Albert et al., 2011). Our phylogeny shows *C. punctatus* from the Rio Araguaia as the sister lineage of the remaining specimens which are distributed throughout the Amazon basin (Fig. 3). This arrangement is congruent to the area cladogram for Neotropical fishes constructed by Albert and Carvalho (2011) under which a clade composed by the Tocantins, Araguaia and Xingu basins is sister to a clade composed by the remaining drainages within the Amazon.

Although Isbrücker and Nijssen (1988) did not report specimens of *Chilodus* from the Rio Araguaia basin, Lowe-McConnell (1991) cited *Chilodus* sp. from a tributary of the Rio das Mortes in the Araguaia drainage. Later, Vari and Ortega (1997) described the occurrence of *C. punctatus* from many Amazonian tributaries including several in the Rio Araguaia basin and Lucinda et al. (2007) reported *C. punctatus* in the middle Rio Tocantins. Additional and more detailed taxonomic studies are required to evaluate whether this lineage of *C. punctatus* from the Rio Araguaia basin merits recognition as a distinct species.

The second clade within the *Chilodus punctatus* species-complex (clade 10) is distributed throughout the Amazon and Guianas. For the most part, our analysis failed to resolve relationships within this clade, but it did group *Chilodus punctatus* from the Rio Juruá and Río Nanay with *C. fritillus* from the Río Madre de Dios in Peru, a tributary of the Rio Mamoré-Madeira (Figs. 2 and 3). These river basins are included in the major ecoregion of the western Amazon and Mamoré-Madre de Dios as detailed by Albert et al. (2011). The close genetic similarity between *C. fritillus* and these western *C. punctatus* (particularly those from the Río Nanay) is striking given the pronounced separation of these regions in linear distance, but much more so in terms of distances along rivers. It further suggests that the intense spotting and the absence of a midlateral stripe that was used to diagnose *C. fritillus* (Vari and Ortega, 1997) may be a function of pronounced regional variation within a chromatically

plastic species. Resolution of this question necessitates in-depth analysis of multiple population samples of the genus from the western Amazon.

The remaining individuals of *C. punctatus* within clade 10 are distributed throughout the Amazon drainage, including the Takutu-Branco system in the northeast of the basin, the Rio Guamá in the east of the system and the Rio Purus in the western Amazon. Notably, the clade also includes a specimen of *C. zunevei* from the independent Atlantic-draining Commewijne River in Suriname northeast of the Amazon basin (Figs. 2 and 3).

If *Chilodus punctatus* is eventually split into multiple species, the lineage marked herein as Clade 10 will be the most likely to bear the original name. The holotype of *C. punctatus* was described from Lake Amuku within the Rupununi Savannas of the upper Essequibo River system (Müller and Troschel, 1844). This locality lies about 50 linear km from the Takutu River in the upper Rio Branco drainage, where two samples of *C. punctatus* used in this analysis originated. During high water periods, the Rupununi Savannas (the so called Rupununi portal) connect the Essequibo River basin (*via* the Rupununi River and the Takutu River) with the Rio Branco, the major tributary of the Rio Negro which is, in turn, the largest northern tributary of the mainstream Amazon. This interconnection allows ichthyofaunal exchange between these otherwise separate Essequibo and Amazon biogeographic provinces (Hubert and Renno, 2006; Souza et al., 2012). Considering that *C. punctatus* has been reported from both the Essequibo and Takutu rivers (Sidlauskas and Vari, 2012; Souza et al., 2012), it is likely that our analyzed specimens from the Takutu River are genetically similar to those living in the type-locality within the upper Essequibo basin.

The taxonomic history of *Chilodus zunevei* is complex and the results of this study further contribute to the uncertainty. *Chilodus zunevei* was originally described as a distinct species by Puyo (1945). That nominal form was soon thereafter reduced to the subspecies level by Géry (1964) who soon thereafter synonymized it into *C. punctatus* (Géry, 1977) only to have the species later resurrected by Isbrücker and Nijssen (1988) (see discussion in Vari and Ortega, 1997). The single available specimen of *C. zunevei* appears as nested within *C. punctatus* and potentially related to specimens from near the type locality of the latter species. Although, it seems possible that *C. zunevei* will again become a junior synonym of *C. punctatus* in an eventual taxonomic revision of *Chilodus*, we prefer to not make a formal taxonomic change on the basis of a single sequenced individual.

Overall, our results suggest that substantial unrecognized diversity exists within the Chilodontidae and that the alpha-taxonomy of both *Caenotropus* and *Chilodus* merits revision. Such efforts should include broader geographic sampling than was possible in the present contribution, as well as renewed attention to the patterns of color variation, squamation and tooth structure that served to diagnose the present species limits within the family. Given the increased ease of integration of comprehensive morphological and molecular datasets afforded by recent and ongoing ichthyological collecting programs and new collaborations, it seems likely that such continent-wide studies will soon reveal greater biodiversity than suspected previously not only among the Chilodontidae but also among other groups of Neotropical freshwater fishes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.09.025.

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