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Phylogenetic relationships of the neon tetras *Paracheirodon* spp. (Characiformes: Characidae: Stethaprioninae), including comments on *Petitella georgiae* and *Hemigrammus bleheri*

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Neon tetras (*Paracheirodon* spp.) are three colorful characid species with a complicated taxonomic history, and relationships among the species are poorly known. Molecular data resolved the relationships among the three neon tetras, and strongly supported monophyly of the genus and its sister taxon relationship to *Brittanichthys*. Additionally, the sister-taxon relationship of the rummy-nose tetras *Hemigrammus bleheri* and *Petitella georgiae* was strongly supported by molecular and morphological data. Therefore, we propose to transfer the rummy-nose tetras *H. bleheri* and *H. rhodostomus* to the genus *Petitella*. Furthermore, *Petitella georgiae* is likely to be a species complex comprised of at least two species.

Keywords: Blood-red tetras, Characids, Neon tetras, Phylogeny, Rummy-nose tetras.

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Os neon tetras (*Paracheirodon* spp.) são três espécies de caracídeos coloridos com uma complicada história taxonômica e as relações entre suas espécies são pouco conhecidas. Dados moleculares resolveram as relações entre os três neons tetras, suportando fortemente a monofilia do gênero e a relação de grupo-irmão com *Brittanichthys*. Adicionalmente, a relação de grupo-irmão entre os rodóstomos *Hemigrammus bleheri* e *Petitella georgiae* foi fortemente suportada por dados moleculares e morfológicos. Portanto, nós propomos transferir os rodóstomos *H. bleheri* e *H. rhodostomus* para o gênero *Petitella*. Além disso, é possível que *Petitella georgiae* seja um complexo de espécies composto por, pelo menos, duas espécies.

Palavras-chave: Caracídeos, Filogenia, Rodóstomo, Tetras neon, Tetras sangue-vermelho.

INTRODUCTION

Among characiforms, Characidae is the most diverse Neotropical fish family, with 1,188 valid species, of which 206 were described in the last ten years (Fricke *et al*., 2020a). Most members of Characidae are small-sized fishes, under < 8 cm standard length (SL), and many are popular aquarium species commonly known as "tetras" (Mirande, 2019).

The genus *Paracheirodon* Géry, 1960 is comprised of three small, brilliantly colored neon tetra species from South America (Weitzman, Fink, 1983) which are popular in the aquarium trade. *Paracheirodon axelrodi* (Schultz, 1956) and *P. simulans* (Géry, 1960) occur in small streams and headwater tributaries of the Negro and Orinoco rivers (Weitzman, Fink, 1983; Marshall *et al*., 2011), while *P. innesi* (Myers, 1936) occurs in blackwater and clearwater streams of the Ucayali-Solimões and Purus rivers (Weitzman, Fink, 1983). *Paracheirodon*, thus, is an emblematic example of a group of Amazonian fishes that are distributed in a biogeographical region known as the "Central Blackwater Amazon" (Dagosta, de Pinna, 2019).

Historically, both *P. innesi* and *P. simulans* were described as species of the genus *Hyphessobrycon* Durbin, 1908 (Myers, 1936; Géry, 1960), while *P. axelrodi* was originally described as a species of the genus *Cheirodon* Girard, 1855 (Schultz, 1956). Géry (1960) established the genus *Paracheirodon* — designating *H. innesi* as its type species — due to its morphological affinities with *Cheirodon axelrodi*, but differing from it by the presence of tricuspid uniserial premaxillary teeth. Consequently, until Weitzman, Fink (1983) performed a taxonomic review of the neon tetras, whereby all of the species were placed within the genus *Paracheirodon*, these three species were in three distinct genera (*Cheirodon, Paracheirodon*, and *Hyphessobrycon*) in two different characid subfamilies (Tetragonopterinae and Cheirodontinae).

The taxonomic review of Weitzman, Fink (1983) provided eight morphological synapomorphies to support the monophyly of *Paracheirodon*. However, relationships among the three species were not well established and the authors did not provide any hypothesis of phylogenetic relationships between *Paracheirodon* and other characid groups, due to a lack of phylogenetic informativeness of the morphological

data available at the time. They also provided one synapomorphy for the clade *P. axelrodi* and *P. innesi*, namely the dorsal placement of the lateral blue body stripe and its posterior termination near the base of the adipose fin, which in *P. simulans* reaches the caudal fin base.

Multilocus (Javonillo *et al*., 2010; Oliveira *et al*., 2011), mitogenome (Yan *et al*., 2017), and total-evidence (Mirande, 2019) phylogenies support the inclusion of *Paracheirodon* in the subfamily Stethaprioninae (also called "Clade C" or "Clade 52" in Javonillo *et al.* (2010) and Oliveira *et al*. (2011), respectively), as well as the sister-species relationship of *P. axelrodi* and *P. innesi*; however, none of these studies included *P. simulans.* These phylogenies also provided new sister-group relationships with species of other genera such as *Brittanichthys axelrodi* Géry, 1965, *Hemigrammus bleheri* Géry, Mahnert, 1986, some species of *Hyphessobrycon* (*H. santae* Eigenmann, 1907, *H. compressus* Meek, 1904, *H. pulchripinnis* Ahl, 1937, *H. eques* Steindachner, 1882, *H. megalopterus* Eigenmann, 1915, *H. erythrostigma* Fowler, 1943, and *H. socolofi* Weitzmann, 1977), which Mirande (2019) refers it as the "true" *Hyphessobrycon*, and *Petitella georgiae* Géry, Boutière, 1964. However, as there are no morphological or molecular phylogenetic studies including *P. simulans*, the monophyly of *Paracheirodon* has never been tested using molecular data. Consequently, the phylogenetic relationships among the three species of the genus have yet to be resolved. Therefore, the aim of the current study was to test the *Paracheirodon* monophyly hypothesis using a molecular phylogenetic approach, through analysis of the mitochondrial genes cytochrome C oxidase subunit I (COI) and the 16S ribosomal RNA (16S rRNA).

MATERIAL AND METHODS

Study area and sample collection. Sampling of *P. simulans* was carried out in palm swamps in an interfluvial region of the middle Negro River, at the headwaters of Igarapé Tulia (0°40'0.12"S, 63°33'51.48"W), during 2009-2010 (see Marshall *et al*., 2011 for a complete field description). We also sequenced five *Brittanichthys axelrodi* specimens from Santa Isabel do Rio Negro (0°36'58" S, 64°55'24" W), due to its close phylogenetic relationship with the genus *Paracheirodon* (Javonillo *et al*., 2010; Mirande, 2019), as well as one individual of *Petitella georgiae* from the Purus River (6°22'30" S, 63°16'29"W), since Mirande (2019) suggests a close phylogenetic relationship of *Petitella* and *Paracheirodon*. Individuals were collected using small dip nets, then preserved in 95% ethanol while in the field, before being deposited posteriorly in the Universidade Federal do Amazonas animal tissue collection (CTGA) using individual ID tags (Tab. 1).

Molecular data. Whole genomic DNA from the muscle tissue of five *P. simulans* individuals was extracted using 2% CTAB solution (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl, 1% PVP) (Doyle, Doyle, 1987), plus 15 mg/mL of Proteinase K. The mitochondrial gene cytochrome C oxidase subunit I (COI) was PCR-amplified using the M13-tailed cocktails FishF2/FishR2 and VF2/VR1d (Ivanova *et al*., 2007), and 16S rRNA using primers 16S-L2508 (5'-CTCGGCAAACATAAGCCTCGCCTGTTTACCAAAAA-3') and 16SH-SLA

TABLE 1 | Specimens analyzed in this study. Species listed are valid names. Collection abbreviations are as follows: CTGA, Coleção de Tecidos de Genética Animal, Universidade Federal do Amazonas, Manaus, Brazil.

Species	Specimen	GenBank accession number	
		16S rRNA	COI
Brittanichthys axelrodi	CTGA 105358	MN971616	MN974145
Brittanichthys axelrodi	CTGA 105360	MN971617	MN974146
Brittanichthys axelrodi	CTGA 105362	MN971618	MN974147
Brittanichthys axelrodi	CTGA 105594	MN971619	MN974148
Brittanichthys axelrodi	CTGA 105595	MN971620	MN974149
Paracheirodon simulans	CTGA 17659_1	MN971621	MN974150
Paracheirodon simulans	CTGA 17659_2	MN971622	MN974151
Paracheirodon simulans	CTGA 17659_3	MN971623	MN974152
Paracheirodon simulans	CTGA 17659_4	-	MN974153
Paracheirodon simulans	CTGA 17659_5	MN971624	MN974154
Petitella georgiae	CTGA 103222	MN971625	MN974155

(5'-TGCACCATTRGGATGTCCTGATCCAA-3') in a total of 15 µL PCR mix, which included 1.5 µL 25 mM $MgCl_2$, 1.5 µL 10 mM dNTPs (2.5 mM each dNTP), 0.5 µL 20 mg/µL Bovine Serum Albumin (BSA), 1.5 µL 10X Taq Buffer plus KCl (100 mM Tris-HCl – pH 8.8 at 25°C – 500 mM KCl, 0.8% (v/v) Nonidet P40), 1.5 µL of primer cocktails (2 pM each), 0.5 µL *Taq* DNA polymerase (1 U/µL), 1.0 µL of template DNA (50 to 100 ng/µL), and 7.0 µL of ddH_20. PCR cycling conditions were as follows: 94°C (30 s), 35 cycles of 94°C (30 s), 50°C (35 s for COI; 40 s for 16S rRNA), and 72°C (90 s) followed by 72°C (5 min). Exonuclease I – Shrimp Alkaline Phosphatase (ExoSAP) was used to purify PCR products that were then used for fluorescent dye terminator sequencing, applying the same PCR primers following the manufacturer's protocols for ABI BigDye Terminator (ThermoFisher). The sequencing reaction products were precipitated using a 100% ethanol/125 mM EDTA solution, which was then resuspended in Hi-Di Formamide, and resolved on ABI 3500XL automatic sequencer (ThermoFisher).

Molecular data of *P. axelrodi*, *P. innesi*, and other characids obtained from GenBank were also included for posterior data analysis (Tab. **S1**). All sequences generated in this study have been deposited in GenBank (Tab. 1).

Data analysis. The nucleotide sequences were organized and verified using Geneious 6 (Kearse *et al*., 2012). The forward and reverse chromatogram reads for each sequenced sample were assembled into contigs and verified visually. The COI nucleotide sequences were also translated into putative amino acids; no internal stop codons were found. Additional COI and 16S rRNA data of *P. axelrodi*, *P. innesi* and other characids from the "clade C" of Javonillo *et al.* (2010) were also included. We also conducted a detailed search in GenBank for additional data of Stethaprioninae *sensu* Mirande (2019) submitted posteriorly to the publications of Javonillo *et al.* (2010) and Oliveira *et al.* (2011). All sequences obtained from GenBank were checked for species misidentification and the taxonomic status of all terminal taxa were evaluated using

Eschmeyer's Catalog of Fishes (Fricke *et al*., 2020b). We used MAFFT v7.07 (Katoh *et al*., 2002) to perform an automatic alignment of COI and 16S rRNA sequences separately, followed by a final visual verification. After alignment, COI and 16S rRNA sequences of each species were concatenated prior to performing a Maximum Likelihood (ML) phylogenetic inference using RAxML 8.1.21 (Stamatakis, 2014). Tree searches were made under the GTRGAMMA substitution model, and the extended majority-rule consensus tree criterion (autoMRE) was used to determine the number of sufficient bootstrap replicates. No partitioning schemes were applied.

RESULTS

A maximum sequence length of 642 bp of the cytochrome C oxidase subunit I and 556 bp of the 16S ribosomal RNA was obtained after the alignment and manual edition of five *P. simulans* samples. For *Brittanichthys axelrodi*, a maximum of 669 bp of the COI and 566 bp of the 16S rRNA was recovered, while *Petitella georgiae* had 536 bp of the COI and 590 bp of the 16S rRNA recovered. The alignment, including other characids, had sequence lengths between 516 and 581 bp for 16S rRNA and 522 to 678 bp for COI, while the concatenated dataset had a total of 1318 bp. Uncorrected p-distance between *P. simulans* and *P. axelrodi*, and *P. simulans* and *P. innesi* COI sequences were 16.6% and 16.2%, respectively, while p-distance between *P. axelrodi* and *P. innesi* was 10.0% (Tab. 2).

The phylogenetic reconstruction using the COI and 16S rRNA concatenated dataset recovered the monophyly of *Paracheirodon* with high bootstrap support (91%), where *P. axelrodi* and *P. innesi* figure as sister species, while *P. simulans* is sister to this clade (Fig. 1). The results also support *Brittanichthys* as the sister-group of *Paracheirodon* (98% bootstrap support). *Petitella georgiae* from Peru and the Purus River were sister taxa, albeit divergent (p-distance = 9.1%), and formed sister clade to *Petitella bleheri* (p-distance = 12.6%); this phylogenetic relationship was highly supported (100% bootstrap support). In contrast, we were unable to confirm the monophyly of the clade comprised of *Paracheirodon*, *Brittanichthys*, *Petitella georgiae* + *Petitella bleheri*, as proposed by Mirande (2019).

TABLE 2 | Uncorrected pairwise p-distance between species of *Paracheirodon*, *Brittanichthys axelrodi*. *Petitella georgiae* and *Petitella bleheri*. Distances were calculated using a 678 bp fragment of the citochrome C oxidase subunit I gene.

Species	1	2	3	4	5	6	7
1. *Paracheirodon axelrodi*	-						
2. *Paracheirodon innesi*	0.100	-					
3. *Paracheirodon simulans*	0.166	0.162	-				
4. *Brittanichthys axelrodi*	0.169	0.167	0.174	-			
5. *Petitella georgiae* Purus	0.183	0.195	0.201	0.151	-		
6. *Petitella georgiae* Peru	0.196	0.193	0.200	0.156	0.091	-	
7. *Petitella bleheri*	0.184	0.184	0.196	0.180	0.126	0.126	-

FIGURE 1 | Maximum Likelihood phylogenetic reconstruction of Stethaprioninae *sensu* Mirande (2018) using 16S ribosomal RNA and cytochrome C oxidase subunit I concatenated dataset after inclusion of *Paracheirodon simulans* and *Petitella georgiae*. Bootstrap values (≥ 75%) are shown near the nodes. Neon tetras are highlighted in red, and rummy-nose tetras are highlighted in blue.

Petitella Géry & Boutière, 1964

Type-species. *Petitella georgiae* Géry & Boutière, 1964

Diagnosis. The genus *Petitella* is readily distinguished from all remaining characid genera by the possession of a distinctively bright red head, the presence of a black horizontal bar that extends from the end of the caudal peduncle to the middle rays of the caudal-fin, and the presence of an oblique black bar in each caudal-fin lobe, separated by white colored bands. Contact between frontals anterior to frontal fontanel present; posterodorsal margin of ethmoid cartilage and lateral ethmoids distant from lateral ethmoids; 17 or fewer branched anal-fin rays; only one or two anal-fin hooks on each ray of adult males; the presence of parallel longitudinal ridges on the posterior field of scales; scales covering one-third of the length of caudal-fin lobes; coloration of the head distinctively red, especially the snout.

Petitella bleheri (Géry & Mahnert, 1986), new combination

Hemigrammus bleheri Géry & Mahnert, 1986:41, fig. unnumb. (original description; type-locality: Middle Rio Negro basin, Brazil, probably near Rio Jufaris).

Petitella rhodostoma (Ahl, 1924), new combination

Hemigrammus rhodostomus Ahl, 1924:405, fig. unnumb. (original description; type-locality: Pará). — Ramsperger, 1924:810 (type-locality description: tributary of the Rio Tapajoz [Tapajós], Santarém, Pará).

Comparative remarks. *Petitella georgiae* is distinguished from its congeners by a long and wide maxillary (*vs*. very short and round in *P. bleheri*, and short in *P. rhodostoma*); single row of premaxillary teeth (*vs*. two in *P. bleheri* and *P. rhodostoma*); dentary with 9–11 teeth with 5 cuspids (*vs*. 6, with 6 or 7 cuspids, followed by 1 or 2 tricuspidate ones in *P. bleheri*, and 5–6 with 5 cuspids, usually followed by 4 conical teeth in *P. rhodostoma*); absence of black spot on lower posterior border of caudal peduncle (*vs*. present in *P. bleheri* and *P. rhodostoma*).

Petitella bleheri is distinguished from its congeners by the much more intense and widespread red color of the head, extending up to the humeral region (*vs*. limited red coloration and not extending to humeral region in *P. georgiae* and *P. rhodostoma*); horizontal black bar on the end of the caudal peduncle is never prolonged forward (*vs*. prolonged up to the anal-fin in *P. georgiae* and *P. rhodostoma*); anal-fin hyaline (*vs*. a black bar on the base of the anterior part of the anal-fin, continuing obliquely on the branched rays in *P. georgiae* and *P. rhodostoma*).

Petitella rhodostoma is distinguished from its congeners by the red head color not extending to the humeral region and the presence of a black spot on the lower posterior border of the caudal peduncle (*vs*. head color not extending to humeral region with only one black spot on caudal peduncle in *P. georgiae*, and head color extending to humeral with two black spots on caudal peduncle in *P. bleheri*); dentary with 5–6 teeth, with 5 cuspids, usually followed by 4 conical ones (*vs*. 9–11 teeth, with 5 cuspids in *P. georgiae*, and 6 teeth, with 6 or 7 cuspids, followed by 1 or 2 tricuspidate ones in *P. bleheri*).

DISCUSSION

Our results support the monophyly of *Paracheirodon*, proposed by Weitzman, Fink (1983) based on morphological data. In addition, the sister-species relationship of *P. axelrodi* and *P. innesi* was also confirmed, which had been suggested by the same authors based on the position and termination of the blue lateral body stripe in the vicinity of the adipose fin.

Numerous analyses published in the last two decades have helped to clarify phylogenetic relationships within Characiformes, including characids (*e.g.* Orti *et al*., 2008, Javonillo *et al*., 2010; Oliveira *et al*., 2011, Thompson *et al*., 2014, Mirande, 2019). The two large-scale phylogenies of Characiformes (Javonillo *et al*., 2010; Oliveira *et al*., 2011) subdivided the Characidae into three principal clades, with Javonillo *et al.* (2010) being the first to reveal the monophyly of *Paracheirodon*, *Brittanichthys*, *Petitella bleheri* and the "true" *sensu* Mirande (2019) species of *Hyphessobrycon*. Using a total-evidence phylogenetic approach, Mirande (2019) found the same clade composition and the same phylogenetic relationships: (((*Paracheirodon, Brittanichthys*), the "*Petitella* clade" [*P. bleheri* + *P. georgiae*]), the "true" *Hyphessobrycon*).

Following Mirande (2019), all of these genera and species share only one morphological synapomorphy: the pelvic-fin bony hooks absent in adult males of species bearing hooks on fins (see Appendix S8, node 988). However, in this study, we were unable to recover phylogenetic relationships other than the sister taxon relationship of *Paracheirodon* and *Brittanichthys,* due to a lack of phylogenetic information in the COI and 16S rRNA genes for deep phylogenetic nodes (Javonillo *et al*., 2010).

The phylogenetic reconstruction (Fig. 1) also confirms *Petitella georgiae* as a sister-group of *Petitella bleheri*. The striking p-distance divergence (9.1%) between the Peruvian *P. georgiae* and our samples from the Purus River indicates the possibility of *Petitella georgiae* being a species complex. The monophyly of *P. bleheri* and *P. georgiae* was highly supported in our analyses — the first time *Petitella georgiae* was included in any molecular phylogeny — as well as by the morphological data of Mirande (2019), who reported seven morphological synapomorphies supporting the sister-taxon relationship between these two species (see Appendix S8, node 1129).

Petitella georgiae, *Petitella bleheri* and *Petitella rhodostoma* (Ahl, 1924) share a very similar coloration marked by an intensely bright red head and the presence of three conspicuous horizontal black bars on the caudal fin (Mirande, 2010). Although Géry, Mahnert (1986) compared the type material of *H. bleheri* to both *H. rhodostomus* and *P. georgiae*, they chose not to discuss either phylogenetic affinities or the validity of the genus *Petitella* itself, due to the "new weights being given to certain cranial characters in the tetras as proposed by certain anatomists", citing Weitzman, Fink (1983). Mirande (2010) stated that "*Petitella georgiae* Géry & Boutière is mainly distinguished from *Hemigrammus bleheri* by having only one row of premaxillary teeth (*vs.* two rows)".

Although *P. rhodostoma* was not included in our analyses nor in Mirande's (2019) matrices, there is little doubt that these three species are very closely related and likely form a monophyletic group. Only the rummy-nose tetras share a distinctive and intensely bright red head, which is of different color and pattern than the rest of the body — a pattern not observed in any other characid species (Mirande, 2019, Appendix S1, character 491). Given that both morphological and molecular data support monophyly of these species, and *Hemigrammus* Gill, 1858 has been demonstrated as

a non-monophyletic entity in multiple studies (Javonillo *et al*., 2010; Oliveira *et al*., 2011; Mirande, 2009, 2010, 2019) and in our study *Hemigrammus unilineatus* Gill, 1858, the type species of *Hemigrammus,* is sister to *Moenkhausia hemigrammoides* Géry, 1965, we therefore suggested the transfer of both *H. bleheri* and *H. rhodostomus* to the genus *Petitella* Géry & Boutière, 1964 and provided a tentative diagnosis for the genus based on original descriptions of the species and the morphological synapomorphies of Mirande (2019). Compelling evidence for the monophyly of the rummy-nose tetras has been published in the recent years using morphological (Lima, Souza, 2009; Mirande, 2010), molecular (this study) and total-evidence (Mirande, 2019) datasets.

By the inclusion of new taxa into characid phylogenies, it was possible to confirm previous hypotheses and propose new ones. However, it is also important to point out that there is still a huge knowledge gap regarding phylogenetic relationships of Characidae, which is likely to remain for some time. For example, of the 141 genera recognized by Mirande (2019), at least 40 do not have any molecular data available in GenBank (pers. obs.) and many genera and species remain *incertae sedis.* This is due to a lack of information beyond their original descriptions, which makes it difficult to infer phylogenetic relationships of these species and genera, which, in turn, guide higher-order classification.

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The authors declare no competing interests.

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