

# Revision of the genus *Prochilodus* (Teleostei: Prochilodontidae) from Bolivia based on a regional analysis of the mtDNA control region

Revisión del género *Prochilodus* (Teleostei: Prochilodontidae) de Bolivia basada en un análisis regional de la región control del ADNmt

## ORIGINAL ARTICLE/ ARTÍCULO CIENTÍFICO

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## ABSTRACT

Fishes of the genus *Prochilodus* are economically and ecologically important migratory detritivorous species widely distributed in the large drainage systems of South America. As they are subject to fishing exploitation and changes due to human disturbances in rivers, we have considered the revision of the genus for Bolivia using morphological and genetic tools. The morphological analysis consisted of 31 morphometric variables, 11 meristic variables, 11 osteological variables and nine coloration variables in 152 specimens from different drainages of Bolivia. The genetic evaluation was based on a Maximum Likelihood phylogenetic analysis of the control region locus (mitochondrial genome) in 39 Bolivian specimens, as well as in *Prochilodus* sequences available in GenBank. The results showed the formation of two specific clades in Bolivia with relative morphological similarity, corresponding to *P. lineatus* and *P. nigricans*. Only one diagnostic morphological character was revealed between these two species, the coloration pattern of the caudal fin. For both species, intraspecific differentiation was evidenced at molecular level. In the case of *P. lineatus*, geographic morphological differentiation was also found. Given the evidence of sympatric independent lineages of

difficult morphological differentiation, local stock management is required to develop conservation strategies in *Prochilodus*.

**Keywords:** Amazon basin, Characiformes, La Plata basin, morphometry, phylogenetics, sábalo

## RESUMEN

Los peces del género *Prochilodus* son especies migratorias detritívoras de gran importancia económica y ecológica, ampliamente distribuidas en los grandes sistemas de drenaje de América del Sur. Debido a que están sujetos a la explotación pesquera y a cambios por disturbios humanos en los ríos, hemos considerado la revisión del género para Bolivia utilizando herramientas morfológicas y genéticas. El análisis morfológico consistió en 31 variables morfométricas, 11 variables merísticas, 11 variables osteológicas y nueve variables de coloración en 152 especímenes de diferentes cuencas de Bolivia. La evaluación genética se basó en un análisis filogenético de Máxima Verosimilitud de la región de control (genoma mitocondrial) en 39 especímenes bolivianos, así como en secuencias de *Prochilodus* disponibles en GenBank. Los resultados mostraron la formación de dos clados específicos en Bolivia con relativa similitud morfológica, correspondientes a *P. lineatus* y *P. nigricans*. Solo se reveló un carácter morfológico diagnóstico entre estas dos especies, el patrón de coloración de la aleta caudal. Para ambas especies, se evidenció diferenciación intraespecífica a nivel molecular. En el caso de *P. lineatus*, también se encontró diferenciación morfológica geográfica. Dada la evidencia de linajes independientes simpátricos de difícil diferenciación morfológica, se requiere un manejo local de los stocks para desarrollar estrategias de conservación en *Prochilodus*.

**Palabras clave:** Cuenca del Amazonas, Characiformes, cuenca del Plata, morfometría, filogenética, sábalo

## INTRODUCTION

*Prochilodus* Agassiz in Spix & Agassiz 1829, one of the most important fish genera in the Neotropics, is comprised of abundant and conspicuous migratory species that form an important fraction of commercial and subsistence fisheries at the continental level (Smolders *et al.* 2000, Carolsfeld *et al.* 2003, Barletta *et al.* 2015). The genus is widely distributed in the large drainage systems of South America, and

is characterized by highly modified lips, teeth and jaws suitable for the consumption of detritus and periphyton (Flecker 1996, Barbarino Duque *et al.* 1998, Castro & Vari 2004). During each annual cycle, these fish form large migratory schools, moving hundreds to thousands of kilometers along the main river channels for breeding and feeding purposes, and in the process intervene in the carbon flow and habitat heterogeneity of the rivers where they transit (Bowen 1983, Flecker 1996, Lucas & Baras 2001, Taylor *et al.* 2006).

Morphologically, *Prochilodus* has a low variability that generates taxonomic uncertainty among its species (Mago-Leccia 1972, Loubens *et al.* 1991, Castro & Vari 2004). Consequently, Castro & Vari (2004) have used diagnostic variables, semi-diagnostic variables and/or a biogeographic character (one species per basin) to discriminate the 13 species considered valid: *P. argenteus* Spix & Agassiz 1829; *P. brevis* Steindachner 1875; *P. britskii* Castro 1993; *P. costatus* Valenciennes in Cuvier & Valenciennes 1850; *P. harttii* Steindachner 1875; *P. lacustris* Steindachner 1907; *P. lineatus* (Valenciennes 1837); *P. magdalenae* Steindachner 1879; *P. mariae* Eigenmann 1922; *P. nigricans* Spix & Agassiz 1829; *P. reticulatus* Valenciennes 1850; *P. rubrotaeniatus* Jardine 1841; *P. vimboides* Kner 1859. This biogeographic character refers to the high vagility of *Prochilodus* in their respective basins, establishing a species per basin (Sivasundar *et al.* 2001). However, notable exceptions have been recorded, such as the São Francisco basin with three described species and the upper Paraná and Uruguay rivers with *P. lineatus* and *P. vimboides* reported in sympatry (Castro & Vari 2004).

Two valid species of *Prochilodus* are reported in Bolivia, where they are commonly known as sábalos: *P. lineatus* in the La Plata basin and *P. nigricans* in the Amazon basin (Reis *et al.* 2003, Carvajal-Vallejos *et al.* 2014, Sarmiento *et al.* 2014). Two other species described for Bolivia were included in the past, *P. beni* Pearson 1924 and *P. labeo* Loubens *et al.* 1991, but are currently considered synonyms of *P. nigricans* (Castro & Vari 2004). Sábalos are major components of commercial, subsistence and indigenous fisheries in Bolivian watersheds (Smolders *et al.* 2000, Van Damme *et al.* 2011, Argote *et al.* 2014). Being subject to extensive fisheries exploitation and continuous anthropogenic changes in rivers (*e.g.*, overexploitation, fragmentation by dams, pollution, among other disturbances), *Prochilodus* species are prime candidates for morphological and genetic revision.

Considering the prominent threats, taxonomic uncertainty and the key role these species play in their ecosystems, there is a need to reevaluate the specific characteristics of *Prochilodus* in Bolivia through molecular phylogenetic (mtDNA) and morphological analysis. It is important to define independent evolutionary units to prevent local extinctions and contribute to sustainable management. Studies of this nature reinforce the need for conservation and help define management actions that mitigate or prevent the loss of biological diversity in Bolivia.

## METHODS

The genetic approach focused on the mitochondrial DNA control region (mtDNA CR) to allow us the recognition of the inter- and intraspecific relationships

of *Prochilodus* by construction of a regional phylogenetic tree. The regional phylogenetic tree constituted by unique haplotypes, some of them present in Bolivia, determined the South American lineages and corroborated which Bolivian lineages were relevant for morphological analysis.

The regional phylogenetic tree was also used to propose two minimum values of nucleotide divergence supported by morphology (Castro & Vari 2004) to discriminate *Prochilodus* species. The species category of 10 taxonomic units currently considered valid was reevaluated.

The morphological analysis was performed in concordance with the phylogenetic analysis and correlated with the values of interspecific discrimination. In addition, a second morphological analysis was performed to determine if there are intraspecific morphological differences by watershed. The genetic and morphological analyses allowed us to recognize the taxonomic units of *Prochilodus* in Bolivia.

### **DNA collection, extraction and sequencing**

The control region (CR) sequences of mtDNA (mitochondrial DNA) were obtained from 48 Bolivian specimens belonging to the Ichthyological Collection ULRA-Museo de Historia Natural "Alcide d'Orbigny" (ULRA-MHNC) in Cochabamba, Bolivia. Thirty-eight *P. lineatus* specimens belong to the río Pilcomayo drainage, eight *P. nigricans* to the río Beni drainage and two *P. nigricans* to the río Madre de Dios drainage.

Each specimen was measured and photographed, and genomic DNA was extracted from a small muscle sample. DNA was obtained by incubating the muscle tissue in 100 µL of 10% Chelex-100 (Bio-Rad), 0.2% SDS and 0.4 mg Proteinase K/ml for two hours at 55 °C, then at 95 °C for 15 min.

The CR of the mtDNA was amplified in a 20.7 µL cocktail containing 0.8 µL dNTPs (8 mM), 4 µL reaction buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.2 µL MgCl<sub>2</sub> (25 mM), 0.96 µL of each primer (10 µM), 0.1 µL (2.5 U) Taq DNA polymerase (Gibco BRL), 1.5 µL DNA template (100 ng/µL) and 11.18 µL H<sub>2</sub>O. PCR conditions were as follows: 94 °C (3 min), 10 cycles of 94 °C (1 min), 50 °C (1 min), 72 °C (1 min), 10 cycles of 94 °C (1 min), 51 °C (30 s), 72 °C (1 min), 10 cycles of 94 °C (1 min), 50 °C (30 s), 72 °C (1 min), followed by 72 °C (2 min). The primers used for amplification (F-TTF and F-12R) and sequencing (PDF2 and PDR2) were those used by Sivasundar *et al.* (2001). Samples were sequenced using the BigDye Terminator kit (Applied Biosystems Inc.) on an ABI 310 automated sequencer following the manufacturer's instructions. All templates were completely sequenced in both directions.

The mtDNA CR consensus sequences were obtained using Chromas version 2.6.5 (Technelysium Pty Ltd, South Brisbane, Queensland, Australia; 2018) and MEGA X version 10.0.5 (MEGA Inc., Englewood, NJ) programs (Kumar *et al.* 2018). Each corresponds to one *Prochilodus* specimen, 38 from the La Plata Basin and 10 from the Amazon Basin.

### **Molecular phylogenetic analysis**

The regional analysis revealed the inter- and intraspecific relationships of 13

species of the family Prochilodontidae. Initially the sequence base consisted of the 48 Bolivian sequences and the 926 sequences available in GenBank (Sayers *et al.* 2019) of the CR (DNAMt) of the family Prochilodontidae: *Ichthyocephalus longirostris* (2), *Prochilodus argenteus* (104), *P. brevis* (6), *P. costatus* (8), *P. lacustris* (5), *P. lineatus* (250), *P. magdalenae* (20), *P. mariae* (34), *P. nigricans* (466), *P. reticulatus* (3), *P. rubrotaeniatus* (17), *Semaprochilodus kneri* (1) and *S. insignis* (10). The entire array was aligned using MUSCLE (Edgar 2004) with MEGA X software (Kumar *et al.* 2018). A minimum length of 874 bp was considered to aggregate the largest number of sequences without reducing variability considerably, so 219 short or questionable sequences were discarded. The number of haplotypes was determined using DNA Sequence Polymorphism 6.12.01 software (Rozas *et al.* 2017) and only unique haplotypes were selected. Detailed information associated with the sequences is given in Supplementary Information 1.

Phylogenetic analysis was conducted using MEGA version X software (Kumar *et al.* 2018). Phylogenetic reconstruction was performed using the Maximum Likelihood (ML) method and a Bootstrap analysis with 100 replicates. The best evolutionary model of nucleotide substitution according to the Bayesian Information Criterion (33226.2) was Tamura 3-parameter (T92) (ML = -11412.1; Ts/Tv = 5.6; A = 0.3; C = 0.3; G = 0.2; T = 0.2), with a discrete Gamma distribution (G = 0.4) and invariant sites (I = 0.3).

Nucleotide divergence (Dxy) (Nei 1987) was estimated with DNA Sequence Polymorphism 6.12.01 software (Rozas *et al.* 2017). This nucleotide divergence allowed the determination of two possible interspecific discrimination values which were employed to disaggregate the species of the family Prochilodontidae in correlation to the previously established morphology (Castro & Vari 2004). The first divergence value considered was the lowest one between two valid species (specific diagnosis of Castro & Vari 2004). On the other hand, considering that the morphological diagnostic characters of some of these valid species are ambiguous (overlapping meristic ranges and an unusual pattern in the scales), the second proposed value of interspecific discrimination was the lowest divergence one between morphologically unambiguous delimited species.

Finally, the intraspecific lineages of the two species present in Bolivia (*Prochilodus lineatus* and *P. nigricans*) were reviewed in detail, with the intention of revealing possible incipient or cryptic species. The topology of the phylogenetic tree and the respective nucleotide divergences were considered.

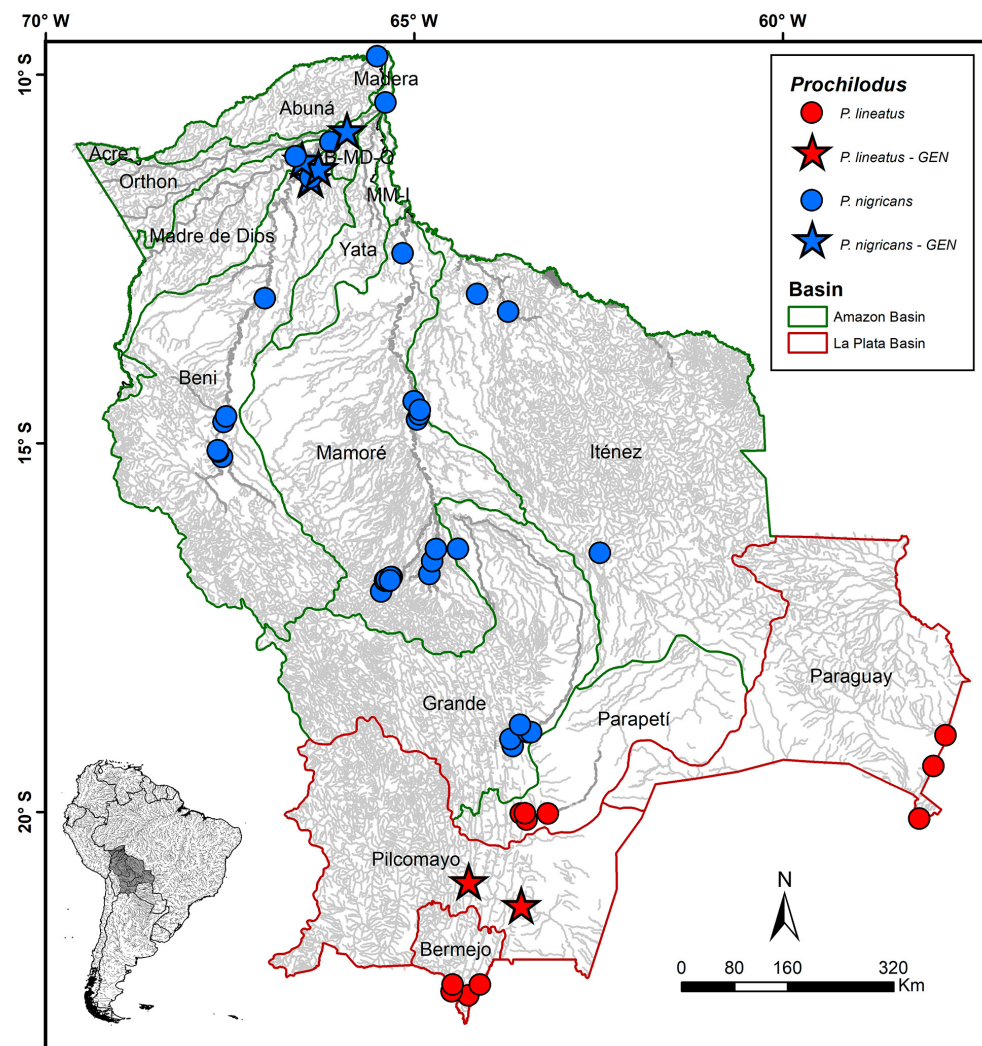
## **Morphological analysis**

The morphological revision was carried out on 152 Bolivian specimens of the genus *Prochilodus* deposited in the Ichthyological Collection ULRA-Museo de Historia Natural "Alcide d'Orbigny" (ULRA-MHNC), Cochabamba, Bolivia. Of the total, 83 specimens come from the main tributaries watersheds of the río Madera, Amazon basin: río Beni (11 localities; 23 specimens), río Iténez o Guaporé (seven localities; 11 specimens), río Madera (two localities; two specimens), río Madre de Dios (three localities; five specimens) and río Mamoré (24 localities; 42 specimens).

The remaining 69 specimens belong to the La Plata basin in Bolivia: río Pilcomayo (two localities; 50 specimens), río Bermejo (four localities; 13 specimens), and three tributaries of the río Paraguay (three localities; six specimens) (Fig. 1). Detailed information associated with the specimens is given in Supplementary Information 2.

The morphological analysis was performed based on 31 morphometric variables, 11 meristic variables, 11 osteological variables and nine coloration variables. The morphometric, meristic and coloration variables followed previous taxonomic reviews of Prochilodontidae: Mago-Leccia (1972), Loubens *et al.* (1991) and Castro & Vari (2004). Two morphometric variables (adipose-fin length and head width) and 10 osteological variables (length of supraoccipital process, length of pelvic bone, number of precaudal vertebrae, number of supraneurals, number of dorsal pterygiophores, number of anal pterygiophores, number of vertebra in contact with the first dorsal pterygiophore, number of vertebra in contact with the last dorsal pterygiophore, number of vertebra in contact with the first anal pterygiophore and

**FIGURE 1.** Fifty-seven localities in Bolivia of the 152 *Prochilodus* specimens examined for phylogenetic and morphological analyses. The blue marks represent the 43 localities of *P. nigricans* and the red marks represent the 13 localities of *P. lineatus*; stars with their respective colors per species represent the 7 localities of the 48 specimens subjected to molecular phylogenetic analyses (GEN). The tributary watersheds of the La Plata basin were Bermejo (4 localities), Paraguay (3) and Pilcomayo (2). Tributaries of the Amazon basin, río Madera drainage, were grouped as follows: Beni (Beni, B-MD-O and Orthon) (11 localities), Madre de Dios (3), Iténez (Iténez and Parapetí) (7), Madera (Abuná and Madera) (2) and Mamoré (Grande, Mamoré, MM-I, Yata) (24). Delimitation of tributary watersheds in the Amazon basin follows Carvajal-Vallejos *et al.* (2014).



number of vertebra in contact with the last anal pterygiophore) were considered for the first time in this study. Morphological variables were analyzed using PAST version 3.16 (Hammer *et al.* 2001).

All morphometric variables were recorded in millimeters (mm) with digital calipers of 0.01 mm precision. These measurements, excluding standard length, were presented as a percentage in two groups: 19 in relation to standard length and 11 in relation to head length. The 11 meristic variables were related to the number of scales and fin rays. The osteological analysis was based on radiographs of 14 specimens. There were 11 osteological variables, the first two were morphometric variables presented in relation to the standard length and the remaining nine were meristic. The observations of external coloration were based on nine variables focused on the patterns of the fins, operculum and body in general.

The first morphological analysis, supported by the phylogenetic tree of Bolivia, was carried out with the purpose of defining the number of taxonomic units of *Prochilodus* in Bolivia. The two species considered valid for Bolivia (*P. lineatus* and *P. nigricans*) were reevaluated using morphometric, meristic, osteological and coloration variables.

The second morphological analysis was carried out to determine the intraspecific morphological variability between the different tributaries of the La Plata basin (Pilcomayo, Bermejo, and Paraguay rivers) and the Amazon basin (Beni, Iténez, Madera, Madre de Dios, Mamoré and Parapetí rivers). All morphometric, meristic and coloration variables were used in the procedure in order to characterize the different geographical samples, which were grouped into tributary watersheds.

## RESULTS

### Molecular phylogenetic analysis

The matrix consisted of 409 unique haplotypes (39 Bolivian sequences and 370 sequences acquired from GenBank; 337 repeated sequences were excluded) with length 874 bp and 309 variable sites. Thirty-two of the thirty-nine Bolivian sequences represented unique haplotypes exclusive to Bolivia (25 *Prochilodus lineatus* and seven *P. nigricans*). The remaining seven sequences (UMSS 13481, UMSS 13489, UMSS 13490, UMSS 13497, UMSS 13536, UMSS 13540, UMSS 13545) were haplotypes shared with specimens from Sivasundar *et al.* (2001), Borden *et al.* (2010), and Penitente *et al.* (2017) from places outside Bolivia.

This ML tree of South American Prochilodontidae sequences (Fig. 2) exhibited the three genera of the family Prochilodontidae (*Ichthyoelephas*, *Semaprochilodus* and *Prochilodus*) as reliably monophyletic stem branches. The clades of *Ichthyoelephas* and *Semaprochilodus* formed outgroups as being external stem branches to the genus under study. The *Prochilodus* clade presented nine of the ten species (*P. argenteus*, *P. brevis*, *P. costatus*, *P. lacustris*, *P. lineatus*, *P. magdalenae*, *P. mariae*, *P. reticulatus*, *P. rubrotaeniatus*) as monophyletic groups with high bootstrap support (77-100%). The exception was *Prochilodus nigricans* which bifurcated into a large

grouping (206 specimens) with moderate bootstrap support (63%) and a small group (four specimens) with high bootstrap support (100%) renamed *P. "nigricans"* for its exclusivity. The basal species of the genus *Prochilodus* were *P. magdaleneae* and *P. reticulatus*. The least divergent species were *P. lacustris*, *P. brevis*, *P. rubrotaeniatus* and *P. "nigricans"*, all hereafter grouped as *P. rubrotaeniatus* complex.

### Nucleotide divergence (Dxy)

The species of the Prochilodontidae family (Tab. 1) presented nucleotide divergence values ranging from 1.93 to 17.23%. Among the clades of the three different Prochilodontidae genera (*Prochilodus*, *Semaprochilodus* and *Ichthyoelephas*), divergences of 9.81 to 17.23% were recorded.

In *Prochilodus*, interspecific divergences ranged from 2.08 to 9.26%. The species most distant from its congeners was *P. magdaleneae* with divergences ranging from 6.82 to 9.26%. The closest species were *P. brevis* and *P. rubrotaeniatus* with a divergence of 2.08%. *Prochilodus lineatus* and *P. nigricans*, the two species present in Bolivia, had a genetic distance of 4.83%.

**TABLE 1.** Genetic divergence among 14 lineages of the family Prochilodontidae consisting of 409 unique mitochondrial DNA Control Region haplotypes. Values were calculated from the average number of nucleotide substitutions per site among lineages (Dxy). Abbreviations correspond to the following lineages: Pni = *P. nigricans*; Pni2 = *P. "nigricans"*; Pla = *P. lacustris*; Pbr = *P. brevis*; Pru = *P. rubrotaeniatus*; Pma = *P. mariae*; Pco = *P. costatus*; Par = *P. argenteus*; Pli = *P. lineatus*; Pre = *P. reticulatus*; Pmq = *P. magdaleneae*; Sin = *S. insignis*; Skn = *S. kneri*; llo = *I. longirostris*.

Lineages	Pni	Pni2	Pla	Pbr	Ru	Pma	Pco	Par	Pli	Pre	Pmq	Sin	Skn	llo
Pni														
Pni2	3.25													
Pla	4.33	2.87												
Pbr	3.76	2.43	2.28											
Ru	4.56	3.67	3.23	2.08										
Pma	3.69	4.05	5.56	5.00	6.05									
Pco	3.86	3.75	5.23	4.19	5.15	4.04								
Par	5.49	6.25	6.90	6.66	7.24	5.36	5.95							
Pli	4.83	4.52	5.47	4.89	5.56	4.72	4.22	5.70						
Pre	6.20	6.71	7.50	6.98	7.80	5.74	6.26	7.00	6.77					
Pmq	7.39	7.77	9.26	7.95	8.76	6.82	6.87	9.22	7.95	7.32				
Sin	9.90	10.39	10.41	10.26	10.41	9.96	9.81	10.69	10.61	11.26	11.62			
Skn	10.41	10.88	11.06	10.26	11.17	10.44	10.05	10.89	11.07	11.26	12.10	1.93		
llo	15.92	15.57	16.37	15.58	16.26	16.17	15.04	17.05	16.60	17.09	17.23	16.53	16.77	



The first value of interspecific discrimination was established by considering the lowest value of divergence between two valid species: 2.08%. Thus, the minor branch of *P. nigricans* called *P. "nigricans"* would be considered a different species and would be closer to *P. brevis* (2.43%) and *P. lacustris* (2.87%) than to *P. nigricans* itself (3.25%).

In order to establish the second value of interspecific discrimination, it was determined that *P. mariae* is the only species of *Prochilodus* in this study that has precise morphological boundaries with each of its congeners. Thus, the second value was the lowest divergence one of this species (vs. *P. nigricans*) which was equal to 3.69%. The species that showed enough genetic divergence to be considered distinct species with this second value were: *P. nigricans*, *P. mariae*, *P. costatus*, *P. argenteus*, *P. lineatus*, *P. reticulatus*, *P. magdalenae*. The species that did not show divergence greater than 3.69% were *P. rubrotaeniatus*, *P. brevis*, *P. lacustris* and *P. "nigricans"*. These four species formed different lineages in the phylogenetic tree (Fig. 2) but were very close to each other and were therefore grouped as the *Prochilodus rubrotaeniatus* complex. This complex of species presented divergences greater than 4.19% with the rest of the species under study and allows a more parsimonious interspecific resolution for the genus *Prochilodus*.

### **Intraspecific lineages of *Prochilodus lineatus***

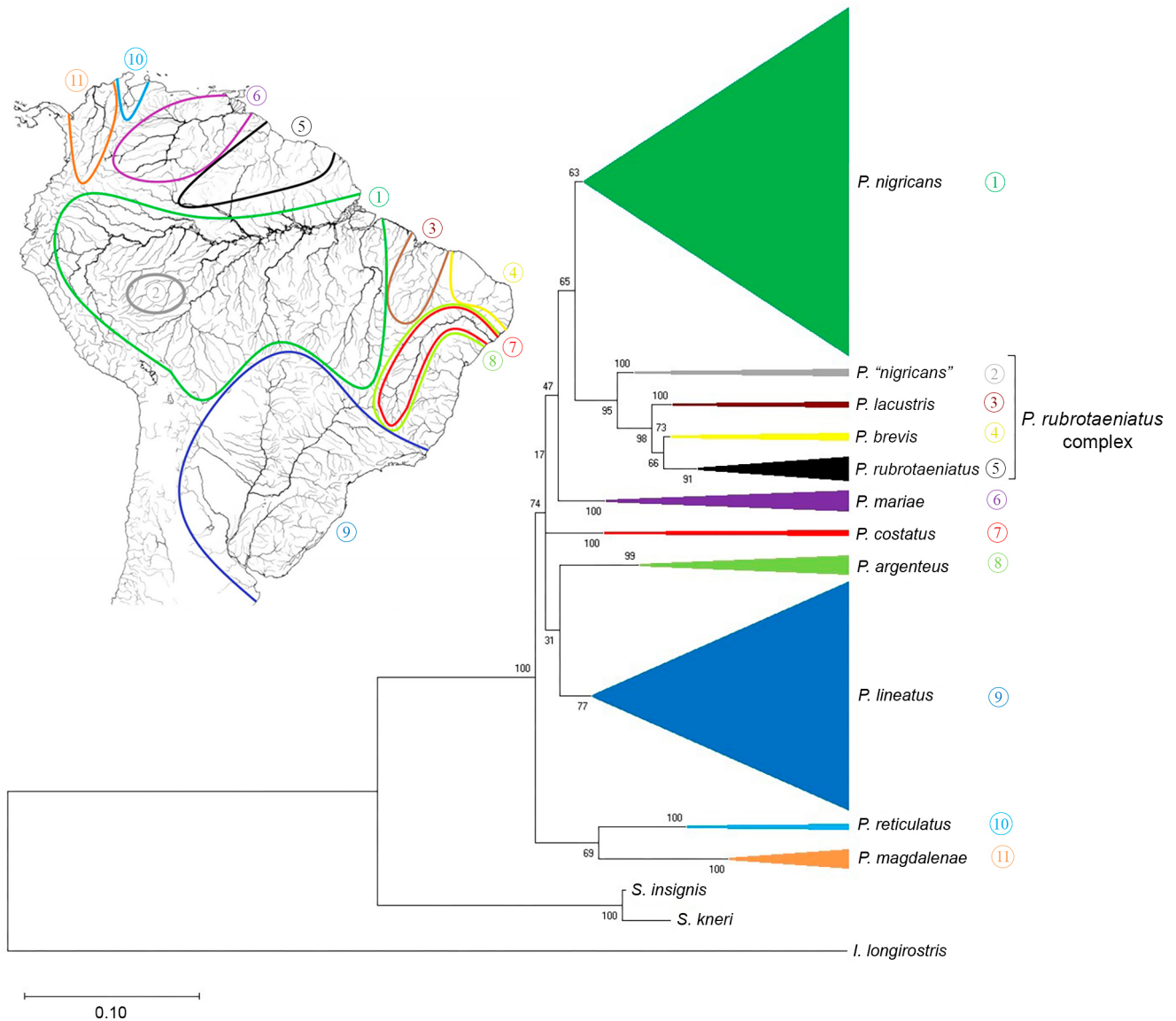
In the South American phylogenetic tree, three clades were identified for *P. lineatus*: *P. lineatus* 1 (110 specimens; black in Fig. 3), *P. lineatus* 2 (14 specimens; red), *P. lineatus* 3 (three specimens; green) (Fig. 3). The clades *P. lineatus* 2 and *P. lineatus* 3 were separated from *P. lineatus* 1 with high bootstraps of 85% and 89%, respectively. In particular, the *P. lineatus* 2 clade was partly constituted by three Bolivian specimens: UMSS 13480 (Río Nuevo, Chuquisaca), UMSS 13529 (Río Pilcomayo, Tarija), UMSS 13533 (Río Pilcomayo, Tarija).

Within the *P. lineatus* 1 clade, some intraspecific lineages (1.2, 1.3, 1.4 and 1.5) were found to present high bootstrap support (78-99%) (Fig. 3). The *P. lineatus* 1.2 lineage included two specimens of Bolivian coded as UMSS 13481 (Río Nuevo, Chuquisaca) and UMSS 13543 (Río Pilcomayo, Tarija). The rest of the Bolivian specimens were included without distinguishable grouping within the clade of *P. lineatus*.

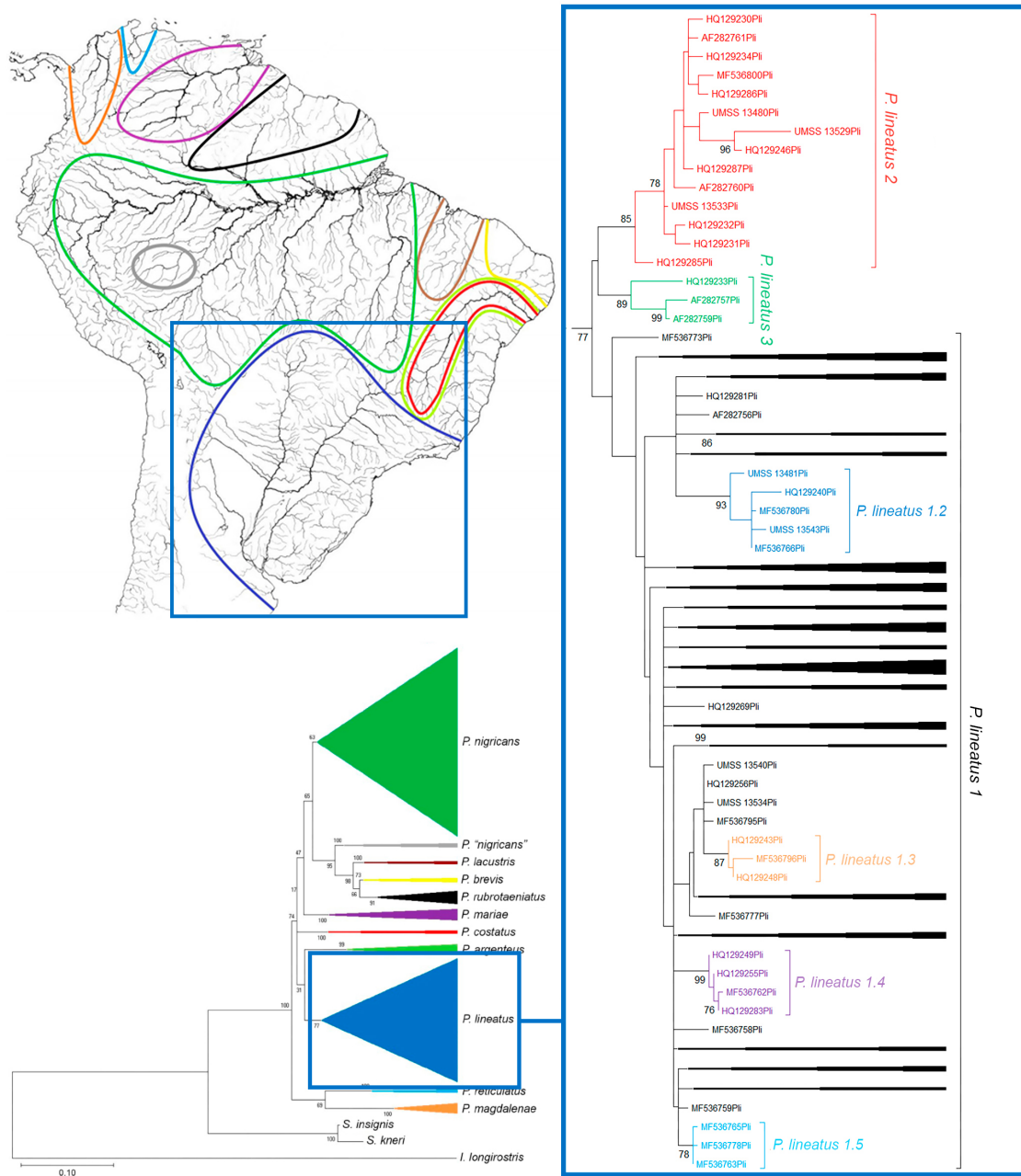
Nucleotide divergence among the clades of *P. lineatus* ranged from 3.13% to 3.48%, with the closest being *P. lineatus* 1 - *P. lineatus* 3 and the most distant *P. lineatus* 1 - *P. lineatus* 2. Using the first value for species disaggregation (2.08%), the three clades could be considered as different species. With respect to the second value of interspecific discrimination (3.69%), none of the three clades would disaggregate so they could not be considered as different species.

### **Intraspecific lineages of *Prochilodus nigricans***

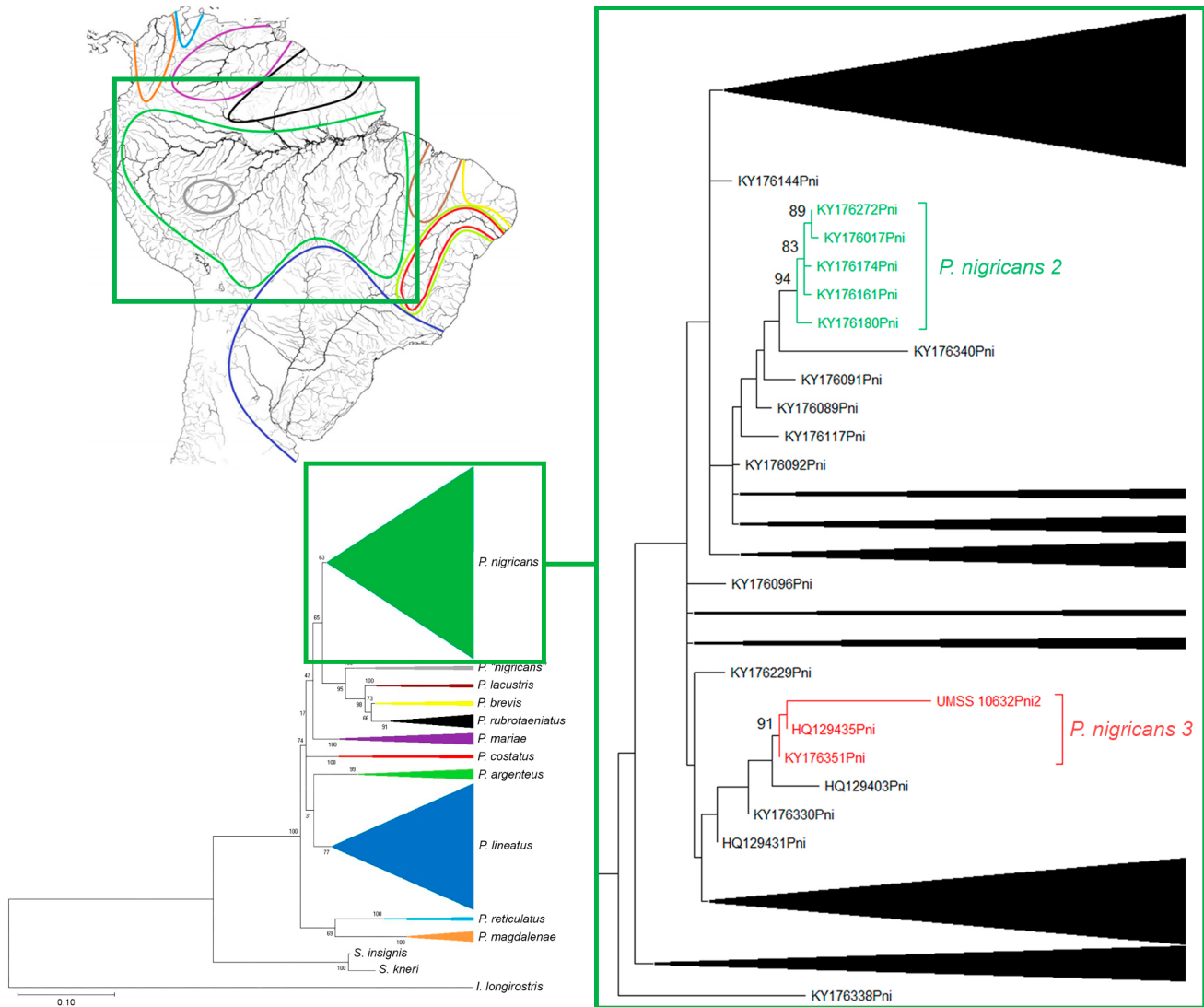
*P. nigricans* in Figure 4 consisted of a single clade that presented two incipient intraspecific lineages: *P. nigricans* 1.2 (five specimens; green; bootstrap 94%) and



**FIGURE 2.** Maximum Likelihood tree of 409 haplotypes of the mitochondrial DNA Control Region corresponding to 13 species of the family Prochilodontidae. The nucleotide substitution model used was Tamura 3-parameter (T92) with a discrete Gamma ( $G = 0.4$ ) distribution and invariant sites ( $I = 0.3$ ) with the following characteristics: transition/transversion bias value = 5.6; base frequencies  $A = 0.3$ ;  $C = 0.3$ ;  $G = 0.2$ ;  $T = 0.2$ . All bootstrap values were exposed below their respective branches. Intraspecific splits were compressed by species. Each species was listed and highlighted by a distinctive color relative to the map in the upper left corner that exposes the distributions of all species considered in this study based on Castro & Vari (2004). The specific clades of *P. "nigricans"*, *P. lacustris*, *P. brevis* and *P. rubrotaeniatus* formed a set called *P. rubrotaeniatus* complex. Two specimens of *Semaprochilodus* (HQ129504; HQ129505) and one specimen of *Ichthyoelephas* (KP025763) from GenBank were used as outgroup.



**FIGURE 3.** Specific clade of *Prochilodus lineatus* consisting of 135 unique mitochondrial DNA Control Region haplotypes. This extended branching was constituted within the South American phylogenetic tree of Figure 2 as depicted in the lower left corner. Only bootstrap values greater than 75% were exposed below their respective branches. Intraspecific clades were listed and highlighted by a distinctive color: *P. lineatus* 1 (black), *P. lineatus* 2 (red), *P. lineatus* 3 (green). In addition, incipient lineages named *P. lineatus* 1.2 (blue), *P. lineatus* 1.3 (orange), *P. lineatus* 1.4 (purple) and *P. lineatus* 1.5 (light blue) were observed within *P. lineatus* 1. In the upper left corner, the geographic distribution of this species is highlighted in blue based on Castro & Vari (2004).



**FIGURE 4.** Specific clade of *Prochilodus nigricans* consisting of 210 unique mitochondrial DNA Control Region haplotypes. This extended branching was constituted within the South American phylogenetic tree of Figure 2 as depicted in the lower left corner. Only bootstrap values greater than 75% were exposed below their respective branches. Intraspecific lineages were listed and highlighted by a distinctive color: *P. nigricans* 1.2 (green) and *P. nigricans* 1.3 (red). In the upper left corner, the geographic distribution of this species is highlighted in green based on Castro & Vari (2004).

*P. nigricans* 1.3 (three specimens; red; bootstrap 91%). The *P. nigricans* 1.3 lineage included one specimen from Bolivia coded as UMSS 10632 (Arroyo Florida, Beni). The remaining Bolivian specimens were included without distinguishable groupings within the *P. nigricans* clade in the South American phylogenetic tree (Fig. 2).

### Morphological analysis

No morphometric, meristic or osteological variables were detected that could be considered diagnostic characters to differentiate the two species of *Prochilodus* recognized as valid for Bolivia (Tabs. 2, 3). The only diagnostic variable was the caudal-fin pattern to differentiate the two species. *P. lineatus* presented a gray to black hyaline caudal-fin, while *P. nigricans* presented a light caudal-fin with a pattern of dark spots forming wavy vertical bars (Fig. 5).

**TABLE 2.** Morphological variation of 31 morphometric characters and 11 meristic characters in *Prochilodus lineatus* and *P. nigricans*.

Variable	<i>P. lineatus</i>	<i>P. nigricans</i>
N	75	77
Standard length	115.24–452.00	70.25–402.00
Percentages of standard length		
Head length	21.73–31.00	25.05–34.37
Body depth	29.65–38.32	26.97–41.19
Predorsal length	36.53–51.88	41.66–51.00
Prepectoral length	20.87–28.43	23.74–34.68
Preanal length	75.88–83.98	73.59–86.93
Prepelvic length	44.21–54.92	43.94–57.79
Dorsal-fin to adipose-fin distance	25.04–33.70	21.05–31.82
Dorsal-fin to caudal-fin distance	39.44–49.79	37.15–49.57
Dorsal-fin base length	12.96–17.35	11.56–18.32
Anal-fin base length	8.56–13.42	8.15–13.20
Dorsal-fin length	22.32–32.05	24.15–38.19
Pectoral-fin length	17.43–23.51	19.06–24.23
Pelvic-fin length	14.74–21.78	10.49–23.62
Adipose-fin length	3.71–7.92	2.85–7.88
Caudal-peduncle length	9.36–15.00	8.75–16.16
Caudal-peduncle depth	10.31–13.97	10.59–13.18
Fork length	105.79–117.15	108.96–118.87
Axillary scale length	1.70–5.49	1.48–10.15
Percentages of head length		
Snout length	40.92–49.09	33.87–50.33
Head width	50.53–63.92	46.30–63.43
Skull length	54.86–70.30	52.48–64.78
Orbital diameter	11.31–20.51	13.97–28.49
Postorbital length	40.91–49.26	39.78–49.30
Interorbital width	47.18–57.91	49.57–63.07
Internarine width	31.25–39.03	31.85–40.54

Prenarine length	25.67–38.67	26.71–39.52
Labial intercomissure width	41.14–51.83	43.09–56.70
Upper lip depth	9.02–19.70	6.16–20.70
Lower jaw length	36.17–46.51	35.37–52.48
Labial precomissure length	33.60–42.32	29.12–44.86
<b>Meristics</b>		
Lateral-line scales	45–51	44–52
Lateral-line (without caudal scales)	40–47	38–47
Perforated caudal scales	0–7	0–8
Superior oblique series	8–10	9–12
Inferior oblique series (anal)	7–9	7–10
Inferior oblique series (pelvic)	8–10	8–10
Unbranched dorsal-fin rays	3	3
Branched dorsal-fin rays	8–10	9–11
Branched anal-fin rays	8–9	8–10
Predorsal scales	15–21	14–22
Circumpeduncular scales	17–23	18–22

**TABLE 3.** Morphological variation of 11 osteological characters of *Prochilodus lineatus* and *P. nigricans*. The lengths of the supraoccipital process and pelvic bone are presented in relation to the standard length of the specimens.

<b>Osteology</b>		
Variable	<i>P. lineatus</i>	<i>P. nigricans</i>
N	4	10
Standard length	217.00–344.00	126.36–402.00
Vertebrae	42–43	42–43
Precaudal vertebrae	17–18	16–18
Supraneurals	4–5	4–5
Dorsal pterygiophores	10	10
Anal pterygiophores	9	9–10
N° Vertebra of the first dorsal pterygiophore	6–9	5–7
N° Vertebra of the last dorsal pterygiophore	14–17	14–16
N° Vertebra of the first anal pterygiophore	25–27	25–27
N° Vertebra of the last anal pterygiophore	30–31	29–33
Supraoccipital process length	7.33–9.69	6.91–12.47
Pelvic bone length	7.46–9.80	7.11–11.65

The morphological analysis included the 75 specimens previously identified as *Prochilodus lineatus*. Eleven morphometric variables were identified to differentiate the specimens in the five watersheds studied: upper lip depth, caudal peduncle depth, interorbital distance, head length, prepectoral length, predorsal length, orbital diameter, fork length, dorsal-fin length, pelvic-fin length and body depth (Fig. 6). The río Pilcomayo drainage was divided into two groups due to obvious morphometric differences between the two capture sites (Pilcomayo 1 = río Nuevo, río Pilaya; Pilcomayo 2 = río Pilcomayo, El Pibe). None of the meristic or coloration variables were concordant with the results of morphometric variation within *P. lineatus*. All specimens presented a gray or dark hyaline caudal-fin, a diagnostic characteristic of the species.

The specimens from the río Paraguay drainage (Upper Paraguay) differed as follows: in the depth of the upper lip of the specimens from the Bermejo (9.02-9.70% vs. 13.04-19.16%), Parapetí (9.02-9.70% vs. 11.10-18.09%) and Pilcomayo 1 (9.02-9.70% vs. 12.92-19.70%) drainages; in the depth of the caudal peduncle (12.31-13.97% vs. 10.31-11.27%) and interorbital distance (54.45-57.91% vs. 50.12-53.23%) of the Parapetí specimens; in head length (26.32-28.42% vs. 21.73-25.65%) and prepectoral length (25.97-27.73% vs. 20.87-25.22%) of the Bermejo specimens; in predorsal length (44.85-47.00% vs. 47.56-54.88%) and interorbital distance (54.45-57.91% vs. 47.18-53.51%) of the Pilcomayo 1 specimens.

Specimens from the río Bermejo drainage differed as follows: in caudal peduncle depth (11.96-13.16% vs. 10.31-11.63%), orbital diameter (11.31-15.38% vs. 16.80-18.93%), head length (21.73-25.65 vs. 26.31-31.00%) and fork length (109.24-111.34% vs. 112.45-117.15%) of the Parapetí specimens; in head length (21.73-25.65% vs. 27.28-30.37%) and prepectoral length (20.87-25.22% vs. 25.63-28.22%) of the Pilcomayo 1 specimens; in upper lip depth (13.04-19.16% vs. 9.33-12.90%) of the Pilcomayo 2 specimens.

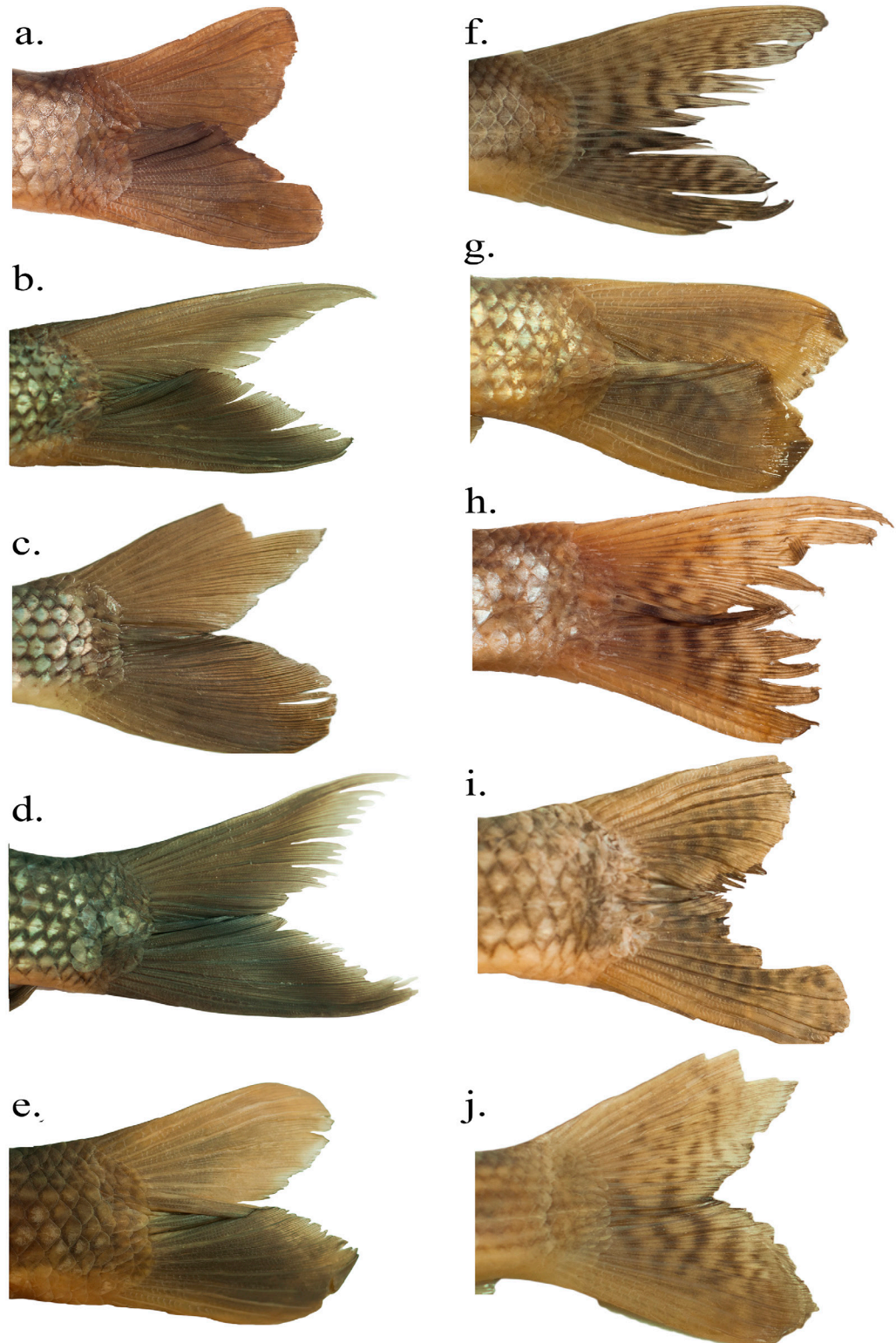
Specimens from the río Parapetí drainage differed in: caudal peduncle depth (10.31-11.63% vs. 12.04-13.71%), orbital diameter (16.80-18.93% vs. 12.72-15.84%), dorsal-fin length (23.22-26.15% vs. 26.85-32.05%) and in pelvic-fin length (16.91-18.37% vs. 19.31-21.78%) of Pilcomayo 1 specimens; in orbital diameter (16.80-18.93% vs. 11.90-16.53%) of Pilcomayo 2 specimens.

Specimens from the Pilcomayo 1 differed from Pilcomayo 2 specimens in upper lip depth (12.92-19.70% vs. 9.33-12.90%) and body depth (32.86-38.21% vs. 29.65-32.66%).

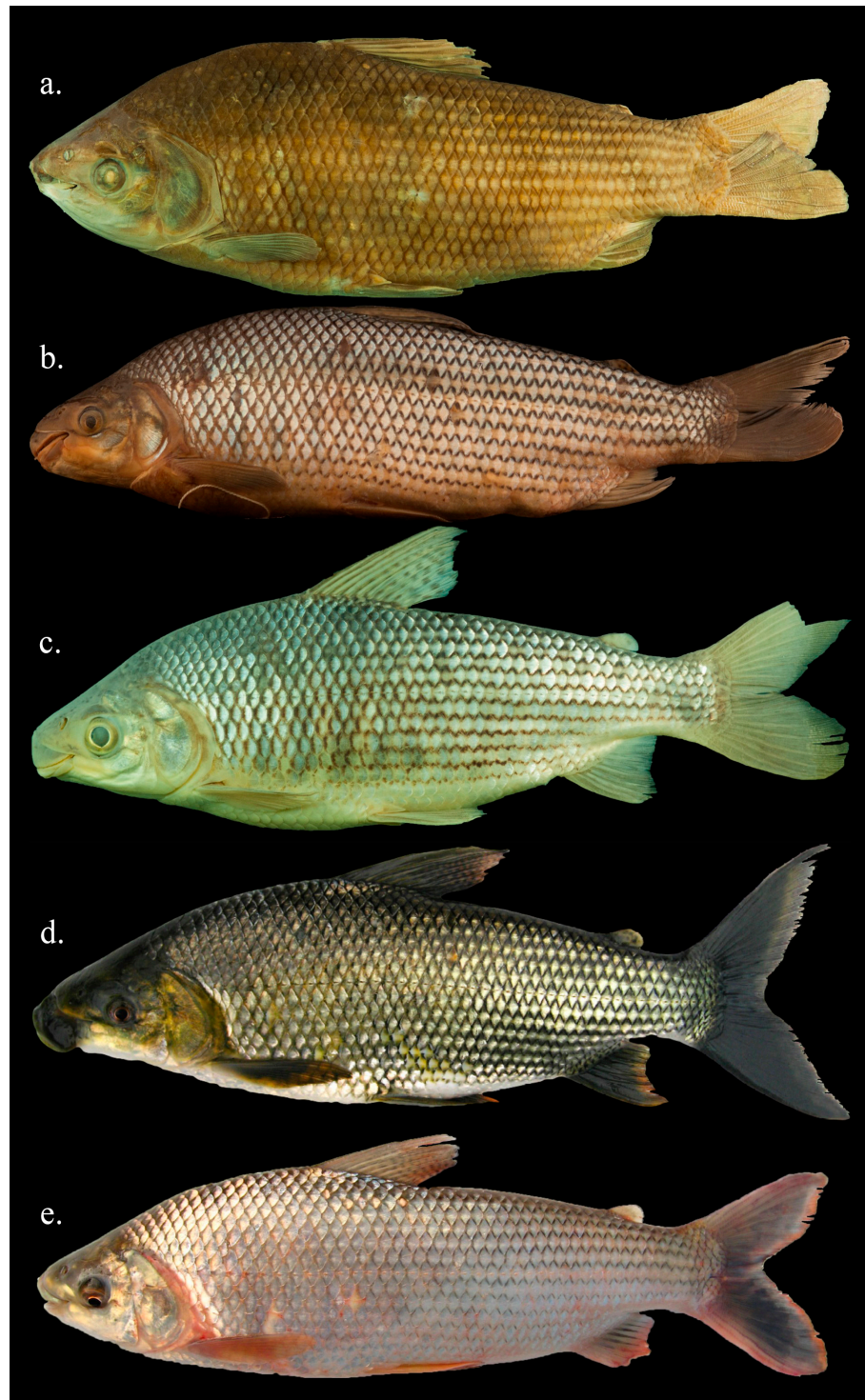
Based on these 11 variables that differentiate the geographic samples, the Principal Component Analysis (PCA; Fig. 7) showed that the first two axes explained 67.4% of the total variation. The first axis (PC1) explained 48.4% of the variation and the second (PC2) 19%. The most influential variables on PC1 were upper lip depth, predorsal length and dorsal-fin length, with eigenvalues of 0.5287, 0.3897 and 0.38946, respectively. The most influential variables on PC2 were orbital diameter and head length, with eigenvalues 0.5038 and 0.37203, respectively.

Unlike *P. lineatus*, there were no morphological differences between the specimens of *P. nigricans* from different tributaries of the Amazon basin in Bolivia (Beni, Iténez, Madera, Madre de Dios and Mamoré watersheds; Fig. 8). All specimens presented the caudal-fin coloration pattern characteristic of the species.

**FIGURA 5.** Variation in caudal-fin coloration pattern in *Prochilodus lineatus*: (a) UMSS 16546, 280.00 mm LE, Laguna Cáceres (río Paraguay); (b) UMSS 13571, 360.00 mm LE, río San Telmo (río Bermejo drainage); (c) UMSS 13832, 159.65 mm LE, río Parapetí (Amazon basin); (d) UMSS 13491, 322.00 mm LE, río Pilaya (río Pilcomayo 1 drainage); (e) UMSS 13544, 299 mm LE, río Pilcomayo (río Pilcomayo 2 drainage); and *Prochilodus nigricans*: (f) UMSS 11055, 158.23 mm LE, Laguna 27 de Mayo (río Beni drainage); (g) UMSS 777, 222.00 mm LE, río Quiser (río Iténez drainage); (h) UMSS 16544, 158.17 mm LE, río Madera (río Madera drainage); (i) UMSS 12770, 402.00 mm LE, San Luis lagoon (río Madre de Dios drainage); (j) UMSS 7315, 128.11 mm LE, río Ichilo (río Mamoré drainage).







**FIGURA 6.** Specimens of *Prochilodus lineatus* from different tributaries of the La Plata basin and a tributary of the Amazon basin: (a) UMSS 16546, 280.00 mm LE, laguna Cáceres (río Paraguay drainage); (b) UMSS 13559, 345.00 mm LE, río Bermejo; (c) UMSS 13832, 159.65 mm LE, río Parapetí (Amazon basin); (d) UMSS 13491, 322.00 mm LE, río Pilaya (río Pilcomayo 1 drainage); (e) UMSS 13536, 242 mm LE, río Pilcomayo (río Pilcomayo 2 drainage)..

## DISCUSSION

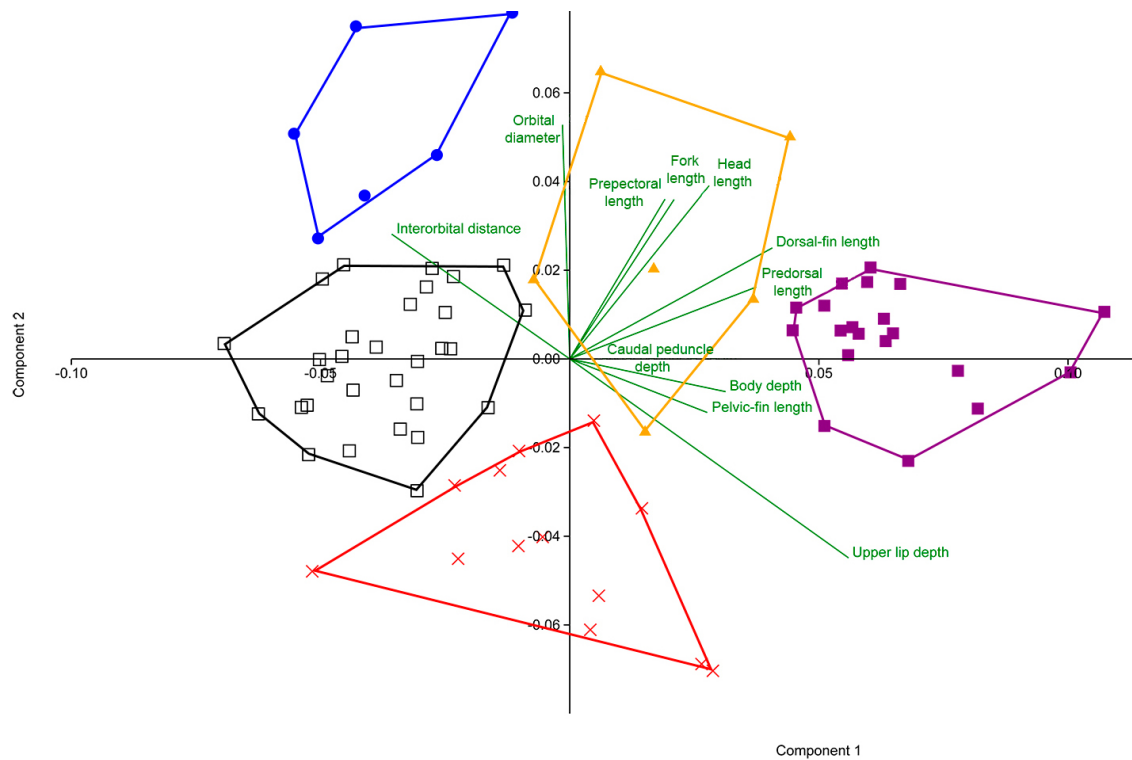
### Phylogenetic and morphological analysis

Two monophyletic clades were observed in Bolivia, which according to the morphological analyses correspond to *P. lineatus* and *P. nigricans*, the two species considered as valid for this country (Lauzanne & Loubens 1985, Castro & Vari 2004, Pouilly *et al.* 2010, Carvajal-Vallejos & Zeballos Fernández 2011, Jégu *et al.* 2012, Carvajal-Vallejos *et al.* 2014, Sarmiento *et al.* 2014, Fricke *et al.* 2022). Nucleotide divergence between *P. lineatus* and *P. nigricans* (4.83%) was higher than the two values of interspecific discrimination (2.08 - 3.69%) considering them distinct species. Morphologically, the disaggregation of these two species was supported by the coloration pattern in the caudal-fin. This character is considered diagnostic for differentiation of these two species, as proposed by Castro & Vari (2004).

The definitive separation of the Amazon and Plata basins with the consolidation of the Michicola Arc in southeastern Bolivia (ca. 11 Ma) (Lundberg *et al.* 1998) probably resulted in an allopatric divergence event in the genus *Prochilodus*. The two species are presumably isolated from each other: *P. nigricans* present in tributaries of the Bolivian Amazon (Beni, Iténez, Madera, Madre de Dios, Mamoré), and *P. lineatus* in tributaries of La Plata basin (Bermejo, Pilcomayo and Paraguay). In addition, the latter species is present in the río Parapetí, which currently runs to the Amazon basin. This river, which flows into the Izozog marshes without reaching the main Amazon system (Latrubesse *et al.* 2012), presents historical connections (paleochannels) with the La Plata basin (río Paraguay drainage) dating back to the Pleistocene (Wilkinson *et al.* 2006), which may explain an ancient capture and the current distribution of *P. lineatus* within the Amazon basin. Sympatry of *P. lineatus* and *P. nigricans* in this watershed is not ruled out.

*Prochilodus lineatus* and *P. nigricans* in Bolivia were morphologically similar. No morphometric, meristic or osteological variables allowed the discrimination between these two species. The only diagnostic character was the caudal-fin coloration pattern: *Prochilodus lineatus* presented a gray to black hyaline caudal-fin, while *P. nigricans* presented a light caudal-fin with a pattern of dark spots forming wavy vertical bars, agreeing with the diagnosis of Castro & Vari (2004) between these two species. The morphological uniformity between these allopatric species of *Prochilodus* possibly spanning an isolation period of 11 Ma (Lundberg *et al.* 1998) supports the hypothesis of morphological stasis for the genus proposed by Castro & Vari (2004). This small degree of morphological differentiation could be due to genetic drift or selective forces during allopatric separation of *Prochilodus* lineages (Melo *et al.* 2016).

Taxonomically, almost all morphological variables of the specimens identified as *P. lineatus* in this study agreed with previous reviews of this species (Loubens *et al.* 1991, Castro & Vari 2004), with the exception of three morphometric variables (snout length, prenarine length, and labial precomissure length) that did not agree with Loubens *et al.* (1991). These morphometric differences are possibly due to modifications in the methods of measurement. A revision of these measurements in the 12 specimens considered by these authors is required.



**FIGURE 7.** Morphological variation of 75 individuals of *Prochilodus lineatus* from different localities of the La Plata and Amazon basins in Bolivia, according to a Principal Component Analysis performed with 11 morphometric variables. Red crosses: Paraguay; blue empty circles: Parapetí; black empty squares: Pilcomayo 1 (río Pilaya, río Nuevo); black squares: Pilcomayo 2 (río Pilcomayo, El Pipe); green crosses: Bermejo.

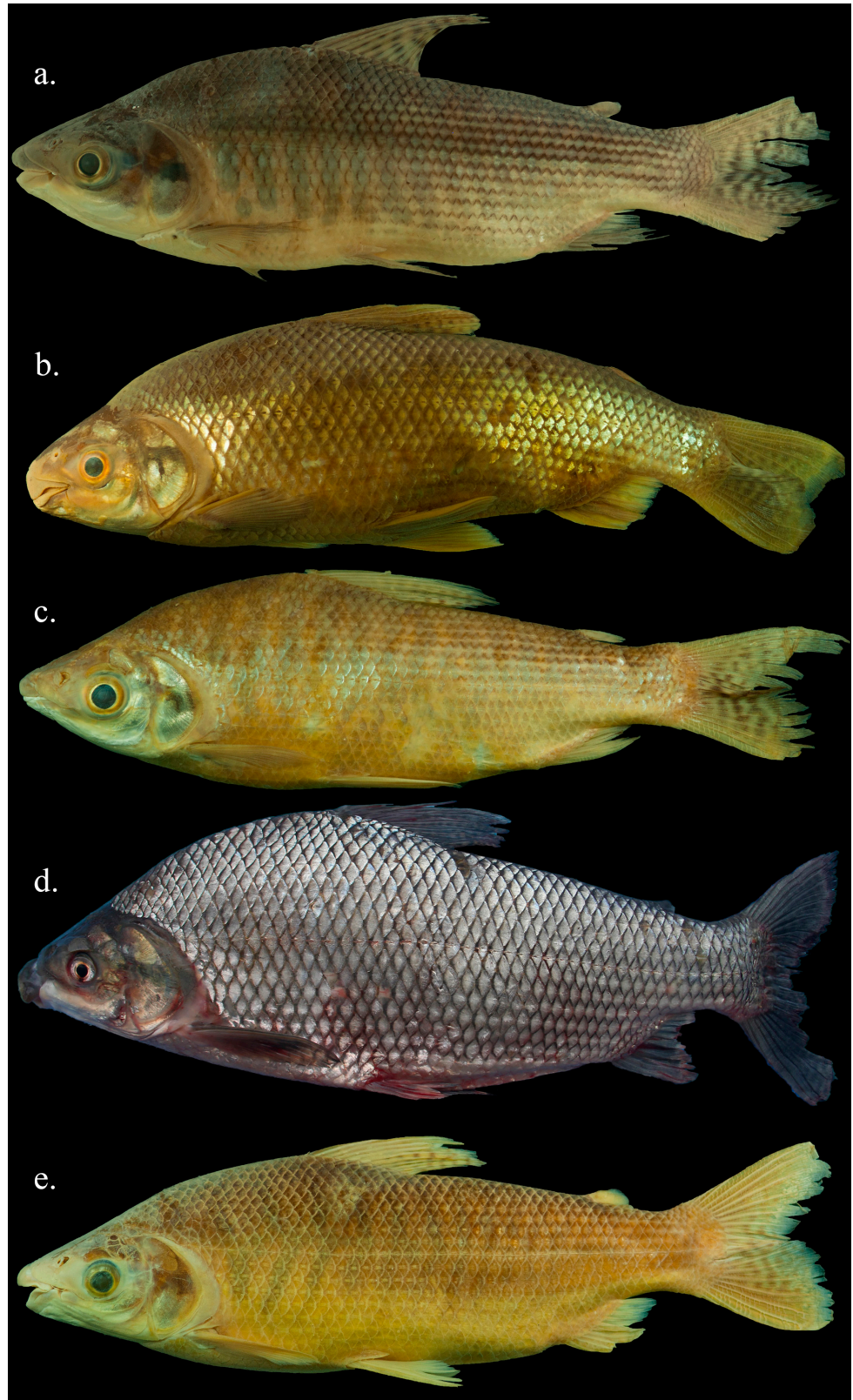
Regarding *P. nigricans*, all ranges of morphological variables coincided with previous publications: Loubens *et al.* 1991, Castro & Vari 2004. Regarding the two species described for Bolivia, *P. beni* and *P. labeo*, their morphological variables coincide with *P. nigricans*. Only three morphometric variables of *P. beni* were outside the range of *P. nigricans*, differing by the bare minimum. This is possibly because the holotype of *P. beni* corresponds to a small juvenile of *P. nigricans*. We consider that the synonymies assignment proposed by Castro & Vari (2004) for *P. beni* and *P. labeo* is correct.

### Intraspecific lineages

For both Bolivian species, *P. lineatus* and *P. nigricans*, some intraspecific differentiation was evident, similar to the results of Sivasundar *et al.* (2001) who noted the presence of divergent haplotypes for the CR (DNAmT) among sympatric samples of *Prochilodus*. Thus, different intraspecific lineages coexist throughout their respective basins, similar to what Landínez-García *et al.* (2020) found for *P. magdalenae*.

For *P. lineatus*, three phylogenetic clades were identified for South America. Two of them were partially conformed by Bolivian specimens. All were considered

**FIGURE 8 .** Specimens of *Prochilodus nigricans* from different tributaries of the Bolivian Amazon: (a) UMSS 11481, 214.00 mm LE, 27 de Mayo lagoon (río Beni drainage); (b) UMSS 2956, 167.00 mm LE, río Blanco (río Iténez drainage); (c) UMSS 16544, 158.17 mm LE, río Madera (río Madera drainage); (d) UMSS 12770, 402.00 mm LE, San Luis lagoon (río Madre de Dios drainage); (e) UMSS 16548, 171.78 mm LE, río Mamoré (río Mamoré drainage).



intraspecific because none showed a divergence greater than the interspecific differentiation detected. Nucleotide divergence values between clades were similar to those recorded by Sivasundar *et al.* (2001) for the same species in the Paraná drainage.

### **Morphological analysis of *Prochilodus* from different tributaries**

In contrast to *Prochilodus nigricans*, the geographical samples of *P. lineatus* could be morphologically characterized with respect to their tributary watershed (Bermejo, Paraguay, Parapetí, Pilcomayo 1, Pilcomayo 2) through eleven morphometric variables. These morphological differences are probably due to ecophenotypic variants related to the resources of each watershed. Cabrera & Candia (1964) mentioned the presence of distinctive body shapes in this species that could be correlated with the biotic and abiotic characteristics of the habitat. Ecological, physiological, environmental and life history traits are required to provide valuable information to elucidate whether these morphological differences correspond to ecophenotypic variations (*e.g.* Römer 2001).

On the other hand, a second explanation for the morphological characterization of *P. lineatus* by watershed could be related to the degree of fidelity of the specimens to their breeding areas. Collins *et al.* (2013) through the chemical composition of otoliths suggest that most individuals of *P. mariae* from a specific collection site share their natal area. Furthermore, Avigliano *et al.* (2017) indicated that otolith microchemistry and morphometry, coupled with scale morphometry, are acceptable habitat markers and represent potential tools to identify areas of larval growth and development. These results merit further study as they could improve our understanding of the connectivity of populations of these species. However, the three lineages present in Bolivia's phylogenetic tree did not present a geographic pattern, presenting congruence with Sivasundar *et al.* (2001), who considered that high vagility of *P. lineatus* within the La Plata basin eliminates genetic differentiation on a geographic scale. Another possibility is the isolation of populations due to homing to natal areas, being that the selection for morphology is strong in these areas but selection against the haplotypes is negligible, a few individuals straying between the spawning areas could homogenize the genetics while keeping the morphology intact.

### **Phylogenetic analysis of Prochilodontidae in South America**

The three branches that emerged in the phylogenetic tree of Prochilodontidae correspond to the genera recognized by Castro & Vari (2004): *Ichthyoelephas*, *Semaprochilodus* and *Prochilodus*. The hypothesis of monophyly of these three genera (Castro & Vari 2004) based on morphological synapomorphies is supported by the phylogram. However, the topology proposed by these authors differs, they propose that *Prochilodus* is a sister genus to the clade of *Semaprochilodus* and *Ichthyoelephas*. Here we propose that *Ichthyoelephas* is a sister group to the *Prochilodus* and *Semaprochilodus* clade, in accordance with the proposal of Melo *et*

*al.* (2016) that was based on a molecular phylogenetic analysis of three mitochondrial loci and three nuclear loci.

The *Prochilodus* clade presented as monophyletic groups the species: *P. argenteus*, *P. brevis*, *P. costatus*, *P. lacustris*, *P. lineatus*, *P. magdalenae*, *P. mariae*, *P. reticulatus*, *P. rubrotaeniatus*, as proposed by Castro & Vari (2004). The monophyly of *P. lineatus* and *P. costatus* agrees with Santos *et al.* (2021) providing strong evidence for both species from analysis of complete mitogenomes, thus differing from that obtained by Melo *et al.* (2016, 2018) nesting *P. lineatus* within the paraphyletic *P. costatus*. The only species that did not come up as a monophyletic lineage was *P. nigricans* which bifurcated into a large grouping considered the proper species and a small group renamed *P. "nigricans"* for its exclusivity. This small group, consisting of four specimens by Machado *et al.* (2017) from the río Purús, probably reflects a genetic divergence within the species. These authors claim that the upper Madera and Purús rivers have diverged genetically from the rest of the *P. nigricans* samples, indicating restricted gene flow between these areas. If this grouping of our South American phylogenetic tree corresponds to this divergent lineage, it would imply that *P. "nigricans"* would also be present in Bolivia. This high degree of population structuring observed in the upper río Madera, which corresponds to the Bolivian system, could be attributed to the presence of rapids scattered along 300 km in its upper course, while the genetic divergence in the upper río Purús could suggest a historical connection between the upper Purús and upper Madera (Machado *et al.* 2017). However, our genetic samples submitted in the phylogenetic analyses were positioned within the major branch considered as *P. nigricans*, none belonged to the small *P. "nigricans"* group. Therefore, both lineages of *P. nigricans* could be present in Bolivia.

The basal branch of the genus *Prochilodus* was formed by *P. magdalenae* and *P. reticulatus*, a topology consistent with Sivasundar *et al.* (2001). This group present in northern South America would represent the first vicariant event for the genus *Prochilodus*, isolating the Magdalena system populations approximately 10 Ma ago (Sivasundar *et al.* 2001). Then *P. magdalenae* would be the most distant species as evidenced in our results by presenting the highest values of nucleotide divergence with respect to its congeners. Melo *et al.* (2018) also recognize the grouping of these two species but consider that *P. magdalenae* remains nested within *P. reticulatus*. In this study these two species formed two monophyletic groupings and presented a nucleotide divergence of 7.32% that far exceeds the two limits (values) of interspecific discrimination, considering them two distinct species.

The least divergent species were *P. lacustris*, *P. brevis*, *P. rubrotaeniatus* and *P. "nigricans"*, grouped here as *P. rubrotaeniatus* complex. Melo *et al.* (2018) consider that two lineages comprising more than one species are present in the Amazon basin: (1) Lineage 6 composed of *P. nigricans* (eastern Amazon), *P. britskii* (upper río Tapajós), *P. brevis* (northeastern Brazil), *P. lacustris* (río Parnaíba River) and *P. rubrotaeniatus* (upper río Orinoco and río Essequibo) and (2) Lineage 7 consisting of *P. rubrotaeniatus* (Suriname), *P. nigricans* (western Amazon). Lineage 6 would correspond to the *P. rubrotaeniatus* complex, since they are formed by the same species and would support the second value of interspecific discrimination that initially grouped these species by presenting reduced nucleotide divergences

between them. The absence of *P. britskii* in our phylogenetic analysis is due to the fact that there are no mtDNA CR sequences available for this species in GenBank (Sayers *et al.* 2019). The only incongruence between Lineage 6 (Melo *et al.* 2018) and the *P. rubrotaeniatus* complex would be the provenance of the specimens identified as *P. "nigricans"* which corresponds to the western Amazon (río Purús drainage), not the eastern Amazon. On the other hand, *P. nigricans* could correspond to Lineage 7 (Melo *et al.* 2018), with no evidence of specimens identified as *P. rubrotaeniatus* within this lineage. We support that the *P. rubrotaeniatus* complex requires a new taxonomic revision as proposed by Melo *et al.* (2016).

The conformation of *P. nigricans* by two differentiated branches casts doubt on its monophyly, suggesting that it comprises more than one species. Melo *et al.* (2016; 2018) also obtained similar results segregating this species into two different lineages. Lopes *et al.* (2020) not only support this bifurcation, but also proposed a third molecular lineage corresponding to *P. nigricans* originating from the río Tapajós drainage. This widely distributed species requires a new and extensive taxonomic revision.

Of the 409 specimens used to build the South American phylogenetic tree, only three specimens did not correlate with previous morphological identification: (1) a specimen of *P. lineatus* (MF536792) was placed within *P. brevis*, (2) a *P. nigricans* (AF282762) within *P. rubrotaeniatus* and (3) a *P. argenteus* (HQ121499) within *P. lineatus*. We consider that these singularities are possibly due to taxonomic missing identification, confusion in the origin of the specimens or alterations in their geographic distributions due to anthropogenic introductions, as occurred in different rivers in eastern and northeastern Brazil (Castro & Vari 2004), and in the río Iténez drainage in Bolivia-Brazil with *Semaprochilodus* (Sarmiento *et al.* 2014).

### **Interspecific discrimination values**

The first value of interspecific discrimination (nucleotide divergence limit of 2.08%) proposed was inconsistent with Castro & Vari (2004) in disaggregating *P. nigricans* into two different species and inconsistent with Melo *et al.* (2016, 2018) in considering *P. "nigricans"*, *P. brevis*, *P. lacustris* and *P. rubrotaeniatus* as distinct species. This value is rejected because it is based on the morphological limits of the 13 valid species (Castro & Vari 2004) that are currently considered doubtful (Melo *et al.* 2016, 2018).

The second value of interspecific discrimination (nucleotide divergence limit of 3.69%) proposed in this study would group the species *P. rubrotaeniatus*, *P. brevis*, *P. lacustris* and *P. "nigricans"* within *P. rubrotaeniatus* complex being consistent with Melo *et al.* (2016, 2018). Likewise, it diverges *P. nigricans* into two different lineages rejecting the proposal of Castro & Vari (2004), and supporting Machado *et al.* (2017). It would also be congruent with the intraspecific range of *P. lineatus* obtained by Sivasundar *et al.* (2001) of 0.3-3.6%. Of the two values proposed, the second one seems the most parsimonious for interspecific resolution in genus *Prochilodus*.

### **Conservation of *Prochilodus***

If we consider that each species of *Prochilodus* has a high genetic diversity and a low population structure, it is tempting to consider that each basin is a single management unit with a reduced risk of overexploitation. However, the identification of *P. nigricans* as an incipient species and *P. lineatus* within the río Amazon basin (río Parapetí drainage) reveals that more than one evolutionary unit may be present in a regional basin. Local stock management is necessary in the development of conservation strategies for sábalo.

The specimens of *P. lineatus* from the río Parapetí watershed presented their own morphological characteristics and possibly form an isolated population, so they should be treated as an exclusive local stock.

It is important to take into account the threats that may disturb or interrupt the migratory routes of sábalo such as physical (*e.g.*, dams) or chemical barriers (*e.g.*, mining contamination). Hydroelectric dams are the main barriers that hinder mass migrations, causing a decrease in recruitment and preventing adults from reaching spawning grounds. Restricted areas could be destined to fishery collapse as they would present a reduced number of adults susceptible to overexploitation (Machado *et al.* 2017). Local extinction of sábalo would cause negative effects on the fisheries value chain, people's livelihoods, local food security and, last but not least, on the ecological role they play within the main tributaries of the Amazon and La Plata basins, among several others (Flecker 1996, Jennings & Kaiser 1998, Taylor *et al.* 2006).

## **SUPELEMENTARY MATERIAL**

Supplementary material 1. Table S1. Mitochondrial DNA control region sequences used in the phylogenetic analysis with their respective GenBank coding.

Supplementary material 2. Examined material of *Prochilodus*

## **ACKNOWLEDGEMENTS**

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## REFERENCES

- Argote A., Van Damme P., Macnaughton A., Villafán S., Carvajal-Vallejos F.M. 2014. Pesca indígena en la Cuenca Amazónica Boliviana: un estudio de caso en la Tierra Comunitaria de Origen Multiétnico II. p 297-338. In: Ministerio de Relaciones Exteriores & Ministerio de Medio Ambiente y Agua (Ed.). Línea de base sobre los ecosistemas y recursos acuáticos en la Amazonía Boliviana. La Paz, Bolivia.
- Avigliano E., Domanico A., Sánchez S., Volpedo A.V. 2017. Otolith elemental fingerprint and scale and otolith morphometry in *Prochilodus lineatus* provide identification of natal nurseries. *Fisheries Research*, 186: 1-10. <https://doi.org/10.1016/j.fishres.2016.07.026>
- Barbarino Duque A., Taphorn D.C., Winemiller K.O. 1998. Ecology of the coporo, *Prochilodus mariae* (Characiformes, Prochilodontidae), and status of annual migrations in western Venezuela. *Environmental Biology of Fishes*, 53: 33-46. <https://doi.org/10.1023/A:1007447004451>
- Barletta M., Cussac V., Agostinho A., Baigún C., Okada E., Catella A., Fontoura N., Pompeu P., Jiménez-Seguro L., Batista V., Lasso C., Taphorn D., Fabre N. 2015. Fisheries Ecology in South American river basins. p 311-348. In: Craig J. (Ed.). *Freshwater Fishes Ecology*. Oxford: Wiley-Blackwell.
- Borden W.C., Li C., Farias I.P., Orti G. 2010. Molecular systematics and biogeography of a South American characiform (*Prochilodus*, Prochilodontidae). Chicago: Department of Biology, Loyola University Chicago.
- Bowen S.H. 1983. Detritivory in Neotropical fish communities. *Environmental Biology of Fishes*, 9: 137-144. <https://doi.org/10.1007/BF00690858>
- Cabrera S.E., Candia C. 1964. Contribución al conocimiento de la biología del sábalo (*Prochilodus platensis* Holmberg) del Río de la Plata; B, Estudio bio-ecológico, III: Análisis del crecimiento; C, Estudio bio-económico. *Revista de Investigaciones Agropecuarias*, 4: 57-83. <http://sedici.unlp.edu.ar/handle/10915/44367>
- Carmo A.O., Brandão Dias P.F.P., Martins A.P.V., Bedore A.G., Kalapothakis E. 2016. Complete mitochondrial genome sequence of *Prochilodus lineatus* (Characiformes, Prochilodontidae), *Mitochondrial DNA*, 27 (3): 1946–1947. [10.3109/19401736.2014.971300](https://doi.org/10.3109/19401736.2014.971300)
- Carolsfeld J., Harvey B., Ross C., and Baer A. 2003. *Migratory Fishes of South America: Biology, Fisheries and Conservation Status*. Victoria: World Fisheries Trust.

- Carvajal-Vallejos F.M., Zeballos Fernández A.J. 2011. Diversidad y distribución de los peces de la Amazonía boliviana. p 101-148. In: Van Damme P.A., Carvajal-Vallejos F.M., Molina Carpio J. (Eds.). Los peces y delfines de la Amazonía boliviana: hábitats, potencialidades y amenazas. Editorial Inia, Cochabamba, Bolivia.
- Carvajal-Vallejos F.M., Bigorne R., Fernández A.J.Z., Sarmiento J., Barrera S., Yunoki T., Pouilly M., Zubieta J., De La Barra E., Jégu M., Maldonado M., Van Damme P., Céspedes R., Oberdorff T. 2014. Fish-AMAZBOL: A database on freshwater fishes of the Bolivian Amazon. *Hydrobiologia*, 732 (1): 19-27. <https://doi.org/10.1007/s10750-014-1841-5>
- Castro R.M.C. 1993. *Prochilodus britskii*, a new species of prochilodontid fish (Ostariophysi: Characiformes), from the rio Apiacá, rio Tapajós system, Mato Grosso, Brazil. *Proceedings of the Biological Society of Washington.*, 106 (1): 57-62.
- Castro R.M.C., Vari R.P. 2004. Detritivores of the South American fish family Prochilodontidae (Teleostei: Ostariophysi: Characiformes): A phylogenetic and revisionary study. Washington DC: Smithsonian Contributions to Zoology, no. 622.
- Chagas A.T.D.A., Carmo A.O., Costa M.A., Resende L.C., Brandão Dias P.F.P., Martins A.P.V., Kalapothakis E. 2016. Description and comparison of two economically important fish species mitogenomes: *Prochilodus argenteus* and *Prochilodus costatus* (Characiformes, Prochilodontidae). *Mitochondrial DNA*, 27 (4): 2852-2853. [10.3109/19401736.2015.1053125](https://doi.org/10.3109/19401736.2015.1053125)
- Collins S.M., Bickford N., McIntyre P.B., Coulon A., Ulseth A.J., Taphorn D.C., Flecker A.S. 2013. Population structure of a Neotropical migratory fish: contrasting perspectives from genetics and otolith microchemistry. *Transactions of the American Fisheries Society*, 142: 1192-1201. <https://doi.org/10.1080/00028487.2013.804005>
- Cuvier G., Valenciennes A. 1850. Histoire naturelle des poissons. Chez F. G. Levrault, Paris. <https://doi.org/10.5962/bhl.title.7339>
- Edgar R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32 (5): 1792-1797. <https://doi.org/10.1093/nar/gkh340>
- Eigenmann C.H. 1922. The fishes of western South America, Part I. The fresh-water fishes of northwestern South America, including Colombia, Panama, and the Pacific slopes of Ecuador and Peru, together with an appendix upon the fishes of the Rio Meta in Colombia. *Memoirs of the Carnegie Museum*, 9 (1): 1-346. <https://doi.org/10.5962/p.234839>
- Flecker A.S. 1996. Ecosystem engineering by a dominant detritivore in a diverse tropical stream. *Ecology*, 77: 1845–1854. <https://doi.org/10.2307/2265788>
- Fricke R., Eschmeyer W.N., Van der Laan R. 2022. *Eschmeyer's Catalog of Fishes: genera, species, references* [Internet]. San Francisco: California Academy of Science, Available from: <http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp>
- Hammer Ø., Harper D.A.T., Ryan P.D. 2001. PAST: Paleontological Statistics software package for education and data analysis. *Palaentologia Electronica*, 4 (1): 9.

- [https://doc.rero.ch/record/15326/files/PAL\\_E2660.pdf](https://doc.rero.ch/record/15326/files/PAL_E2660.pdf)
- Jardine W. (Ed.). 1841. The Naturalists' Library. Ichthyology Vol. 3. W. H. Lizars. Edinburgh. <https://doi.org/10.5962/bhl.title.15849>
- Jégu M., De Queiroz L.J., Camacho Terrazas J., Torrente-Vilara G., Carvajal-Vallejos F.M., Pouilly M., Yunoki T., Zuanon J.A.S. 2012. Catálogo de los peces de la cuenca Iténez (Bolivia y Brasil), p 113-156. In: Van Damme P.A., Maldonado M., Pouilly M., Doria C.R.C. (Eds.). Aguas del Iténez o Guaporé: recursos hidrobiológicos de un patrimonio binacional (Bolivia y Brasil). Editorial INIA. Cochabamba, Bolivia.
- Jennings S., Kaiser M.J. 1998. The effects of fishing on marine ecosystems. *Advances in Marine Biology*, 34: 201-352. [https://doi.org/10.1016/S0065-2881\(08\)60212-6](https://doi.org/10.1016/S0065-2881(08)60212-6)
- Kner R. 1859. Zur Familie der Characinen. III. Folge der Ichthyologischen Beiträge, Denkschriften der Kaiserlichen Akademie der Wissenschaften in Wien, Mathematisch-Naturwissenschaftliche Classe, 17: 137-182.
- Kumar S., Stecher G., Li M., Knyaz C., Tamura K. 2018. MEGAX: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, 35 (6): 1547-1549. <https://doi.org/10.1093/molbev/msy096>
- Landínez-García R.M., Alzate J.F., Márquez E.J. 2016. Mitochondrial genome of the Neotropical trans-Andean fish *Ichthyoelephas longirostris*, Steindachner 1879 (Characiformes, Prochilodontidae). *Mitochondrial DNA*, 27 (3): 2194-2195. <https://doi.org/10.3109/19401736.2014.982622>
- Landínez-García R.M., Narváez J.C., Márquez E.J. 2020. Population genetics of the freshwater fish *Prochilodus magdalenae* (Characiformes: Prochilodontidae), using species-specific microsatellite loci. *PeerJ*, 8:e10327 <https://doi.org/10.7717/peerj.10327>
- Latrubesse E.M., Stevaux J.C., Cremon E.H., May J.H., Tatumi S.H., Hurtado M.A., Bezada M., Argollo J.B. 2012. Late Quaternary megafans, fans and fluvio-aeolian interactions in the Bolivian Chaco, Tropical South America. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 356-357: 75-88. <https://doi.org/10.1016/j.palaeo.2012.04.003>
- Lauzanne L., Loubens G. 1985. Peces del Río Mamoré. Éditions de l'ORSTOM. Paris.
- Lopes U., Galetti P.M. Jr., Domingues de Freitas P. 2020. Hidden diversity in *Prochilodus nigricans*: A new genetic lineage within the Tapajós River basin. *PLoS ONE*, 15 (8): e0237916. <https://doi.org/10.1371/journal.pone.0237916>
- Loubens G.L., Lauzanne L., Géry G. 1991. Contribution à la systématique des *Prochilodus boliviens* (Pisces, Characiformes, Prochilodontidae). *Revue d'Hydrobiologie Tropicale*, 24 (3): 217-239. <https://www.documentation.ird.fr/hor/fdi:35857>
- Lucas M.C., Baras E. 2001. Migration of Freshwater Fishes. Blackwell Science Ltd. Oxford.
- Lundberg J.G., Marshall L.G., Guerrero J., Horton B., Malabarba M.C.S.L., Wesselingh F. 1998. The Stage for Neotropical fish diversification: history of tropical South American Rivers. p 14-48. In: Malabarba L.R., Reis R.E., Vari R.P., Lucena Z.M.S., Lucena C.A.S. (Eds.). Phylogeny and classification of Neotropical fishes, Edipucrs, Porto Alegre, Brazil.

- Machado V.N., Willis S.C., Teixeira A.S., Hrbek T., Farias I.P. 2017. Population genetic structure of the Amazonian black flannelmouth characin (Characiformes, Prochilodontidae: *Prochilodus nigricans* Spix & Agassiz, 1829): contemporary and historical gene flow of a migratory and abundant fishery species. *Environmental Biology of Fishes*, 100 (1): 1-16. <https://doi.org/10.1007/s10641-016-0547-0>
- Mago-Leccia F. 1972. Consideraciones sobre la sistemática de la familia Prochilodontidae (Osteichthyes, Cypriniformes) con una sinopsis de las especies de Venezuela. *Acta Biológica Venezuelica*. Venezuela, 8 (1): 35-96.
- Melo B.F., Sidlauskas B.L., Hoekzema K., Frable B.W., Vari R.P., Oliveira C. 2016. Molecular phylogenetics of the Neotropical fish family Prochilodontidae (Teleostei: Characiformes). *Molecular Phylogenetics and Evolution*, 102: 189-201. <https://doi.org/10.1016/j.ympev.2016.05.037>
- Melo B.F., Dorini B.F., Foresti F., Oliveira C. 2018. Little divergence among mitochondrial lineages of *Prochilodus* (Teleostei, Characiformes). *Frontiers in Genetics*, 9 (170). <https://doi.org/10.3389/fgene.2018.00107>
- Nei M. 1987. *Molecular Evolutionary Genetics*. Columbia Univ. Press. New York.
- Pearson N.E. 1924. The Fishes of the Eastern Slope of the Andes, I: The Fishes of the Rio Beni Basin, Bolivia, Collected by the Mulford Expedition. *Indiana University Studies*, 11 (64): 1-83. [https://www.pecesdebolivia.com/443/biblioteca/documentos/Pearson\\_1924\\_Fishes\\_Eastern\\_slope\\_Andes\\_rio\\_Beni.pdf](https://www.pecesdebolivia.com/443/biblioteca/documentos/Pearson_1924_Fishes_Eastern_slope_Andes_rio_Beni.pdf)
- Penitente M., Vera M., Martinez P., Porto-Foresti F. 2017. Genetic variability and population structure of *Prochilodus lineatus* within Paraná basin. *Ciencias Biológicas*, Universidade Estadual Paulista – UNESP, São Paulo.
- Pouilly M., Jégu M., Camacho Terrazas J., Quintanilla Palacios M., Miranda Chumacero G., Zubieta Zubieta JP., Yunoki T. 2010. Lista actualizada y distribución de los peces en las tierras bajas de la Amazonía Boliviana. *Revista Boliviana de Ecología y Conservación Ambiental*, 28: 73-97.
- Reis R.E., Kullander S.O., Ferraris C.J. Jr. (Eds.). 2003. *Check List of the Freshwater Fishes of South and Central America*. Edipucrs, Porto Alegre.
- Ribeiro J.M., Oliveira R.S., Carmo F.M.S., Yazbeck G.M. 2017. Molecular diversity in mitochondrial genomes for the Characiformes order. Universidade Federal de Sao Joao del Rei, Pca. Frei Orlando.
- Römer U. 2001. Influence of temperature on fertility, growth rates, and reproductive success on selected species of *Apistogramma* (Teleostei, Cichlidae). *Verhandlungen der Gesellschaft fuer Ichthyologie*, 2: 87-106.
- Rozas J., Ferrer-Mata A., Sánchez-DelBarrio J.C., Guirao-Rico S., Librado P., Ramos-Onsins S.E., Sánchez-Gracia A. 2017. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. *Molecular Biology and Evolution*, 34 (12): 3299-3302. <https://doi.org/10.1093/molbev/msx248>
- Santos R.P., Melo B.F., Yazbeck G.M., Oliveira R.S., Hilário H.O., Prosdócimi F., Carvalho, D.C. 2021. Diversification of *Prochilodus* in the eastern Brazilian Shield: Evidence from complete mitochondrial genomes (Teleostei, Prochilodontidae). *Journal of Zoology, Systematics and Evolution Research*, 0: 1–11. <https://doi.org/10.1111/jzs.12475>

- Sarmiento J., Bigorne R., Carvajal-Vallejos F.M., Maldonado M., Leciak E., Oberdorff, T. 2014. Peces de Bolivia. Plural editores, IRD-BioFresh (EU), La Paz, Bolivia.
- Sayers E.W., Cavanaugh M., Clark K., Ostell J., Pruitt K.D., Karsch-Mizrachi I. 2019. GenBank. Nucleic Acids Research., 47(D1): D94–D99. <https://doi.org/10.1093/nar/gky989>
- Sivasundar A., Bermingham E., Ortí G. 2001. Population structure and biogeography of migratory freshwater fishes (*Prochilodus*: Characiformes) in major South American rivers. *Molecular Ecology*, 10: 407–417. <https://doi.org/10.1046/j.1365-294X.2001.01194.x>
- Smolders A.J.P., Van der Velde G., Roelofs J.G.M. 2000. El Niño caused collapse of the Sábalo fishery (*Prochilodus lineatus*, Pisces: Prochilodontidae) in a South American River, *Naturwissenschaften*, 87: 30-32. <https://doi.org/10.1007/s001140050004>
- Spix J.B., Agassiz L. 1829. Selecta genera et species piscium quos in itinere per Brasiliam annis MDCCCXVII-MDCCCXX jussu et auspiciis Maximiliani Josephi I. Bavariae Regis Augustissime peracto collegit et pingendos curavit Dr. J.B. de Spix, digessit, descripsit et observationibus anatomicis illustravit Dr. L. Agassiz, praefatus est et edidit itineris socius Dr. F. C. Ph. de Martius. Typis C. Wolf, Munich.
- Steindachner F. 1875. Die Süßwasserfische des südöstlichen Brasilien. *Sitzungsberichte der Kaiserlichen Akademie der Wissenschaften. Mathematisch-Naturwissenschaftliche Classe*, 70 (1): 499-538.
- Steindachner F. 1879. Zur Fisch-Fauna des Magdalenen-Stromes. *Denkschriften der Kaiserlichen Akademie der Wissenschaften in Wien, Mathematisch-Naturwissenschaftliche Classe*, 39: 19-78.
- Steindachner F. 1907. Über zwei neue Arten von Süßwasserfischen aus dem Stromgebiete des Parnahyba. *Anzeiger der Kaiserlichen Akademie der Wissenschaften, Wien, Mathematisch-Naturwissenschaftliche Klasse*, 44 (10): 152-155.
- Taylor B.W., Flecker A.S., Hall R.O. 2006. Loss of a harvested fish species disrupts carbon flow in a diverse tropical river. *Science*, 313: 833-836. [10.1126/science.1128223](https://doi.org/10.1126/science.1128223)
- Valenciennes A. 1837. Poissons (plates). In: d’Orbigny, A. (Ed.). *Voyage dans l’Amérique méridionale*. Chez Pitois-Levrault et ce., libraires-éditeurs, rue de la Harpe, Paris. <https://www.biodiversitylibrary.org/page/49211037>
- Van Damme P., Carvajal-Vallejos F.M., Rua A., Córdova L., Becerra P. 2011. Pesca comercial en la Cuenca Amazónica Boliviana. p 247-292. In: Van Damme P., Carvajal-Vallejos F.M., Molina J. (Eds.). *Peces y Delfines de la Amazonía Boliviana: hábitats, potencialidades y amenazas*. Editorial INIA, Cochabamba, Bolivia.
- Wilkinson M.J., Marshall L.G., Lundberg J.G. 2006. River behavior on megafans and potential influences on diversification and distribution of aquatic organisms. *Journal of South American Earth Sciences*, 21: 151-172. <https://doi.org/10.1016/j.jsames.2005.08.002>