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Integrative cytogenetic and molecular evidence reveals a new cryptic lineage within the "*Parodon suborbitalis* complex" (Characiformes: Parodontidae)

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Parodontidae is a Neotropical fish family arranged in *Saccodon*, *Parodon*, and *Apareiodon* genera. Despite morphological similarities in some paired species, genetic and chromosomal markers have been useful for resolving taxonomic uncertainty in Parodontidae. *Parodon* representatives inhabiting the Amazonian basin are among the species with subtle morphological features that require accurate identification. Therefore, this study compared *Parodon* species using cytogenetic and genetic markers, with the aim of molecular species delimitation. Additionally, two *Parodon* populations from the Amazon River basin, morphologically identified as *Parodon* sp. (Peixe River) and *Parodon* cf. *buckleyi* (Branco River), were cytogenetically and molecularly characterized. Both *Parodon* sp. and *P.* cf. *buckleyi* presented a 2n = 54 and chromosome markers similar to those of *Parodon nasus*, but with species-specific differences. Genetic distance, Bayesian analysis, and molecular species delimitation methods recognized *P. guyanensis*, *P. pongoensis*, *P. hilarii*, *P. caliensis*, *P. suborbitalis*, *P. magdalenensis*, *P. apolinari*, *P. nasus*, *P.* cf. *buckleyi*, and *Parodon* sp. as valid species. Integrative cytogenetic and molecular data analysis points to *Parodon* sp. (Peixe River) as a Molecular Operational Taxonomic Unit in Parodontidae. These results reveal hidden diversity within *Parodon* and suggest that the "*P. suborbitalis* complex" is non-monophyletic, with distinct groups arranged within a biogeographic framework.

Keywords: Chromosomes, DNA Barcoding, FISH, Integrative taxonomy, MOTU.

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Parodontidae é uma família de peixes Neotropicais agrupada nos gêneros *Saccodon*, *Parodon* e *Apareiodon*. Apesar das similaridades morfológicas em alguns pares de espécies, marcadores genéticos e cromossômicos têm sido úteis para resolver incertezas taxonômicas em Parodontidae. Representantes de *Parodon* da bacia Amazônica estão entre as espécies com características morfológicas sutis que exigem identificação precisa. Assim, este estudo comparou espécies de *Parodon* usando marcadores citogenéticos e genéticos, com o objetivo de delimitar molecularmente as espécies. Adicionalmente, duas populações de *Parodon* da bacia Amazônica, morfologicamente identificadas como *Parodon* sp. (rio do Peixe) e *Parodon* cf. *buckleyi* (rio Branco) tiveram caracterização citogenética e molecular. Ambas, *Parodon* sp. e *P.* cf. *buckleyi* apresentaram 2n = 54 e marcadores cromossômicos similares a *Parodon nasus*, mas com diferenças espécie-específicas. A distância genética, a análise Bayesiana e os métodos moleculares de delimitação de espécies reconheceram *P. guyanensis*, *P. pongoensis*, *P. hilarii*, *P. caliensis*, *P. suborbitalis*, *P. magdalenensis*, *P. apolinari*, *P. nasus*, *P.* cf. *buckleyi* e *Parodon* sp. como espécies válidas. A análise integrativa dos dados citogenéticos e moleculares indica *Parodon* sp. como uma Unidade Taxonômica Operacional molecular. Esses resultados revelam uma diversidade oculta no gênero *Parodon* e sugerem que o "complexo *P. suborbitalis*" não é monofilético, com grupos distintos em uma estrutura biogeográfica.

Palavras-chave: Cromossomos, DNA Barcoding, FISH, MOTU, Taxonomia integrativa.

INTRODUCTION

The Amazon basin is the largest river basin on Earth, covering approximately 6.3 million km^2 (Milliman, Farnsworth, 2011). It supports the highest freshwater biodiversity (Tisseuil *et al*., 2013), with approximately 2,400 valid species (Jézéquel *et al*., 2020), accounting for nearly 15% of all known freshwater fish species worldwide (Tedesco *et al*., 2017). However, the true extent of fish diversity in the Amazon basin is likely underestimated, as numerous new species continue to be described every year (Antonelli *et al*., 2018; Jézéquel *et al*., 2020).

Parodontidae is a Neotropical fish family comprising 32 valid species (Fricke *et al*., 2025), grouped into three genera: *Parodon* Valenciennes, 1849, *Saccodon* Kner, 1863, and *Apareiodon* Eigenmann, 1916 (Pavanelli, 2003). This family has a broad geographic range in South America and Panama, except for some coastal Atlantic basins and Patagonia (Pavanelli, Britski, 2003). Their species are classified into genera based on characters such as jaw lateral teeth and the number of undivided rays in the pectoral fin (Pavanelli, 2003). For species identification, body coloration patterns with a single regular black longitudinal stripe or vertical bars can be used, along with the shape and number of cusps of the symphysial tooth, among others (Pavanelli, 1999).

Among *Parodon* species, the "*P. suborbitalis* complex" was initially proposed by Pavanelli (1999) based on morphological similarities, particularly body color patterns featuring a black zigzag lateral band on a light background, despite inhabiting different

river basins. The species complex originally comprised five species: *Parodon bifasciatus* Eigenmann, 1912, *P. buckleyi* Boulenger, 1887, *P. hilarii* Reinhardt, 1867, *P. nasus* Kner, 1859, and *P. suborbitalis* Valenciennes, 1850. In a taxonomic review of *Parodon* species from Colombia, Londoño-Burbano *et al*. (2011) agreed with the previously proposed "*P. suborbitalis* complex" but suggested a new arrangement. They included one species previously excluded by Pavanelli (1999), *Parodon carrikeri* Fowler, 1940, with a black lateral band with a diffuse zigzag on a dark background, in addition to *P. alfonsoi* Londoño-Burbano, Román-Valencia & Taphorn, 2011, *P. atratoensis* Londoño-Burbano, Román-Valencia & Taphorn, 2011, and *P. magdalenensis* Londoño-Burbano, Román-Valencia & Taphorn, 2011, which were described after the earlier work. The species complex now encompasses nine of the fourteen valid *Parodon* species. Despite detailed morphological descriptions, species identification in the complex relies on average character values with some overlap, making geographic origin an important consideration for identification (see Pavanelli, 1999, and Londoño-Burbano *et al*., 2011 for further details). Most of "*P. suborbitalis* complex" representatives lack genetic and cytogenetic data, which could contribute to resolving this group's classification.

Six *Parodon* species are documented in Brazilian river basins: *P. bifasciatus*, *P. buckleyi*, *P. hilarii*, *P. moreirai* Ingenito & Buckup, 2005, *P. nasus*, and *P. pongoensis* (Allen, 1942) (Pavanelli, 2003; Londoño-Burbano *et al*., 2011). In the Brazilian Amazon River basin, *P. bifasciatus* is found in the northern region, while *P. buckleyi* occurs in the southern part of the basin (Pavanelli, 2003; Londoño-Burbano *et al*., 2011). However, according to Pavanelli (1999), Brazilian populations of *P. buckleyi* from the Machado River sub-basin in Rondônia state are considered isolated representatives of the species, due to numerous waterfalls in the Machado River, which likely serve as geographical barriers to the gene flow. The *P. buckleyi* specimens analyzed by Pavanelli (1999) from the Machado River sub-basin were more robust, with projections of the main black longitudinal band not very evident, in addition to a lower average number of cusps in the premaxillary teeth, in comparison to *P. buckleyi* from Ecuador and Peru.

Cytogenetically, Parodontidae has a conserved diploid chromosome number (2n) of 54 among the species (Bellafronte *et al*., 2011). However, their species can be distinguished according to chromosomal characteristics: karyotypic formula, differential accumulation and number of heterochromatic bands, presence or absence of sex heteromorphic chromosomes, number of sites of rDNAs and snDNAs, distribution and sites number of the pPh2004 satellite DNA, and the W*Ap* repetitive fraction distribution on the karyotypes (Bellafronte *et al*., 2011; Schemberger *et al*., 2011; Ziemniczak *et al*., 2014; Traldi *et al*., 2016, 2020; Santos *et al*., 2019; Nirchio *et al*., 2021; Azambuja *et al*., 2022a,b, 2023). The occurrence of ZW sex chromosome differentiation in some species is a significant feature in the Parodontidae fish's diversification (Schemberger *et al*., 2011, 2019; Oliveira *et al*., 2024, 2025). Species lacking heteromorphic sex chromosomes or with sex chromosomes at different levels of differentiation, such as proto-sex chromosomes, ZW sex chromosomes, and with multiple systems of heteromorphic sex chromosomes (ZZ/ZW_1W_2) were observed in the group (Schemberger *et al*., 2011; Bellafronte *et al*., 2012; Traldi *et al*., 2016, 2020; Nascimento *et al*., 2018; Santos *et al*., 2019; Nirchio *et al*., 2021; Wolf *et al*., 2024). For species in "*P. suborbitalis* complex", just *P. hilarii* and *P. nasus* were assessed by cytogenetic studies (Centofante *et al*., 2002; Vicente *et al*., 2003). However, remarkable karyotype differences are found, *i.e*., *P. hilarii*,

2n = 54 m/sm, ZZ/ZW sex chromosome system, non-syntenic 45S and 5S rDNA sites, and 14–16 pPh2004 satellite DNA sites; *P. nasus*, 2n = 54, 48m/sm + 6st, proto-sex chromosome, 8 pPh2004 satellite DNA sites, among others (Bellafronte *et al*., 2011; Schemberger *et al*., 2011).

The Molecular Operational Taxonomy Unit (MOTU) represents clusters of DNA sequences that differ from each other by more than an established threshold of similarity and are therefore proposed as operational taxonomic units (Floyd *et al*., 2002; Blaxter, 2004). For this purpose, molecular analyses using the Cytochrome c Oxidase subunit I (COI) gene can help in the initial delimitation of species (Hebert *et al*., 2003) and have been widely used in the identification and delimitation of Neotropical fishes (Serrano *et al*., 2018; Ramirez *et al*., 2020; Morais-Silva *et al*., 2023; Souza *et al*., 2023; Almeida *et al*., 2024), including Parodontidae (Bellafronte *et al*., 2013; Nascimento *et al*., 2018; Santos *et al*., 2019; Traldi *et al*., 2020), as well as in fish complex groups (Terán *et al*., 2020; Limeira Filho *et al*., 2024; Fernandes *et al*., 2025). Different modern methods for delimiting species have been proposed for single-locus analyses (Pons *et al*., 2006; Fujisawa, Barraclough, 2013; Zhang *et al*., 2013; Puillandre *et al*., 2021). However, recent analyses have shown that combining other methods with integrative taxonomy yields more representative results for the lineages (Tang *et al*., 2014; Kapli *et al*., 2017; Luo *et al*., 2018; Serrano *et al*., 2018; Traldi *et al*., 2020).

The integration of chromosome markers (cytotaxonomy) and COI sequence analysis has been utilized as a tool to address taxonomy uncertainties among parodontids, especially in species with similar morphological and chromosomal traits (Bellafronte *et al*., 2013; Nascimento *et al*., 2018; Santos *et al*., 2019; Traldi *et al*., 2020; Azambuja *et al*., 2022a). Regarding species in the *Parodon* genus, with special attention to those within the "*P. suborbitalis* complex" a more comprehensive collection of cytogenetic and genetic data is still required. To elucidate the genetic diversity and taxonomic boundaries within *Parodon*, we cytogenetically and molecularly characterized two Amazonian *Parodon* species exhibiting morphological traits, particularly body color pattern, consistent with the "*P. suborbitalis* complex". These data were integrated with chromosomal and mitochondrial COI information from additional congeners, both within and outside the complex, and from different hydrographic regions of the Amazon basin. By applying molecular species delimitation methods within an integrative taxonomic framework, we aimed to assess the occurrence of MOTUs and refine the limits of the "*P. suborbitalis* complex".

MATERIAL AND METHODS

Biological samples. Fifteen individuals (1 female, 8 males, and 6 sex undetermined) previously identified as *Parodon* sp. (Fig. 1A) from the Peixe River, Amazon basin, Tapajós River sub-basin, Pará State, Brazil, 08°39'15.5"S 55°09'24.3"W (Fig. 1C), and thirty-two individuals (10 females and 22 males) identified as *P.* cf. *buckleyi* (Fig. 1B) from the Branco River, Amazon basin, Madeira River sub-basin, Rondônia State, Brazil, 11°55'51.1"S 62°09'09.4"W (Fig. 1C) were analyzed.

Specimen identification was conducted based on geographic origin and color pattern, as this species complex cannot currently be distinguished solely by morphological traits. Specimens were deposited as vouchers in the Ichthyological Collection at Núcleo de

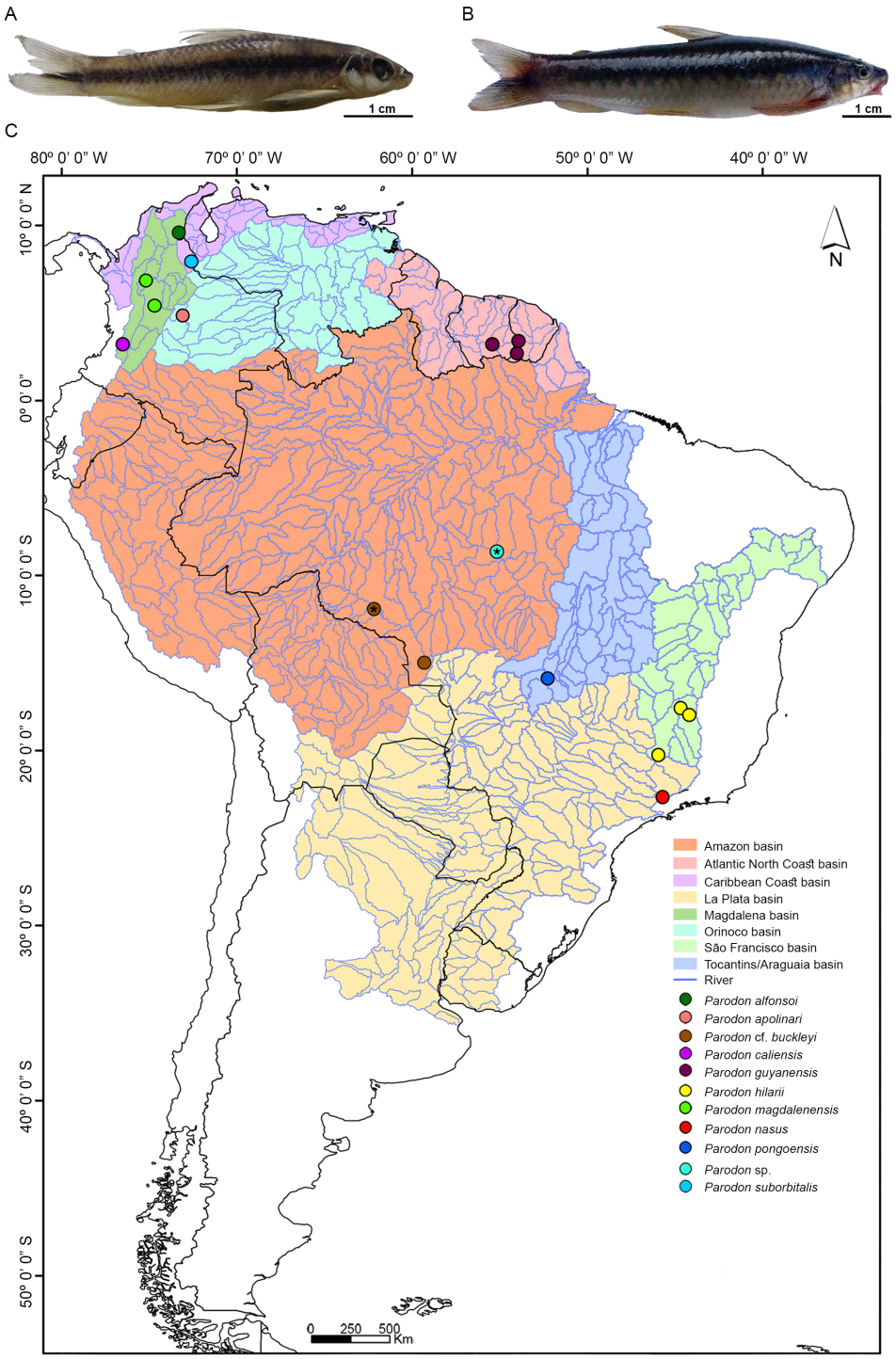

FIGURE 1 | **A.** *Parodon* sp. and **B.** *Parodon* cf. *buckleyi*. **C.** Partial map of South America showing collection sites and river basins for analyzed *Parodon* species. *Correspond to *Parodon* sp. and *P.* cf. *buckleyi* populations analyzed cytogenetically.

Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupelia) of Universidade Estadual de Maringá, Paraná State, Brazil (NUP 22248, 23214, and 23216). Eight liver samples of *Parodon pongoensis* from the Taquaralzinho River, Tocantins-Araguaia basin, Mato Grosso State, Brazil, 15°53'28"S 52°14'56"W (Fig. 1C) and four of *P. hilarii* from the Araras River, São Francisco basin, Minas Gerais state, Brazil, 20°16'15"S 45°55'39"W (Fig. 1C) from the tissue bank of the Laboratório de Citogenética Evolutiva of the Universidade Federal de São Carlos were used for the molecular analyses. In addition, one tissue sample of *P. buckleyi* from the Pindaituba River, Amazon basin, Madeira River sub-basin, Mato Grosso State, Brazil, 15°00'41"S 59°17'18"W (Fig. 1C), from the ichthyological collection of the Museu de Ciências e Tecnologia of the Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre (PUCRS) (Voucher number: MCP 38672) was used for comparisons with our *P.* cf. *buckleyi* samples and included in the molecular analyses.

Chromosomal preparations and classical cytogenetics analysis. Mitotic chromosomes were obtained from the animals' kidneys as described by Bertollo *et al*. (2015). Chromosomes were stained with 5% Giemsa in phosphate buffer (pH = 6.8) to determine 2n, fundamental number (FN), and karyotype formula. Heterochromatic regions were identified by C-banding, as described by Sumner (1972). Metaphases were analyzed and photographed using a bright-field microscope (Olympus BX43) coupled to a DP72 CCD camera (Olympus). Homologous chromosomes were paired and organized into karyotypes following Levan *et al*. (1964). At least 30 metaphases of each species were analyzed to confirm the 2n and heterochromatic bands.

Molecular cytogenetics analysis. Fluorescence *in situ* hybridization (FISH) was performed following Pinkel *et al*. (1986), and six sequences of repetitive DNAs were physically mapped in *Parodon* sp. and *P.* cf. *buckleyi*: 18S and 5S rDNAs, pPh2004 satellite DNA, (GATA)n, and the transposable elements *Helitron* and *Tc1-Mariner*. The 18S probe was obtained according to Hatanaka, Galetti Jr. (2004), and labeled with Biotin-16-dUTP (Biotin-Nick Translation Mix; Roche Applied Science). The 5S rDNA sequence was obtained and labeled with digoxigenin-11-dUTP (DIG-11-dUTP; Jena Bioscience) by polymerase chain reaction (PCR) using genomic DNA from *P. nasus* and the *primers* 5SA and 5SB (Martins, Galetti, 1999). The pPh2004 probe was prepared according to Vicente *et al*. (2003) and labeled with digoxigenin-11-dUTP (Dig Nick Translation Mix; Roche Applied Science) and Biotin-16-dUTP (Biotin-Nick Translation Mix; Roche Applied Science). The (GATA)n probe was obtained according to Traldi *et al*. (2013), and the *Helitron* and *Tc1-Mariner* probes were obtained according to Schemberger *et al*. (2019) and labeled with digoxigenin-11-dUTP (Dig Nick Translation Mix; Roche Applied Science). FISH was performed under stringent conditions (~80%) (300 ng of each probe, 50% formamide, 10% dextran sulfate, 2xSSC, 37°C for 16 h). Signal detection was carried out using Streptavidin Alexa Fluor 488 (Molecular Probes) and anti-digoxigenin-rhodamine (Roche Applied Science). Chromosomes were stained with DAPI (0.2 µL/mL) present in Vectashield mounting medium (Vector) and analyzed in an epifluorescence microscope (Leica DM 2000) coupled to a DFC3000 G CCD camera (Leica). At least 20 metaphases per probe were analyzed to determine FISH signals.

Molecular analysis. Genomic DNA of five individuals of *Parodon* sp., six of *P.* cf. *buckleyi*, eight of *P. pongoensis*, and four of *P. hilarii* were extracted from liver samples following the CTAB (cetyltrimethylammonium bromide) method of Murray, Thompson (1980). DNA was used to amplify the barcode region of the COI by PCR using the *primers* Fish F1 and Fish R1 (Ward *et al*., 2005). Reaction mix contained: 1x *Taq* Reaction buffer (200 mM Tris pH 8.4, 500 mM KCl), 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each *primer*, 1 U *Taq* DNA polymerase (Invitrogen), and 40 ng of DNA. The following reaction program was used: initial denaturation for 10 min at 94°C, 35 cycles of 94°C for 1 min, 54.5°C for 45 sec, and 72°C for 90 sec, and a final extension at 72 °C for 10 min. The PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare) and sequenced on an ABI-prism 3500 Genetic Analyzer (Applied Biosystems).

Electropherograms were reviewed, the nucleotide sequences were corrected in Geneious v. 7.1.9 (Kearse *et al*., 2012) and deposited in GenBank. COI sequences of the species *Parodon alfonsoi* (1), *P. apolinari* Myers, 1930 (3), *P. caliensis* Boulenger, 1895 (1), *P. guyanensis* Géry, 1960 (9), *P. hilarii* (3), *P. magdalenensis* (3), *P. nasus* (5) and *P. suborbitalis* (1) were mined from the Barcode of Life Database (BOLD) (Ratnasingham, Hebert, 2007) and GenBank database (Benson *et al*., 2013) (Tab. **S1**), and were aligned with the sequences of *Parodon* sp., *P.* cf. *buckleyi*, *P. hilarii*, and *P. pongoensis* (this study) using the algorithm ClustalW, integrated with the software Geneious. Sequences were separated into groups, and genetic distances were calculated using MEGA X (Kumar *et al*., 2018), under the Kimura-2-parameters (K2P) evolution model with 1,000 bootstrap replications. The number of haplotypes was verified in DnaSP v5 (Librado, Rozas, 2009), and a haplotype network was generated using the Minimum Spanning Network criterion (Bandelt *et al*., 1999) in PopArt v. 1.7 (Leigh, Bryant, 2015).

One *Leporinus piau* Fowler, 1941 COI sequence (HM405030.1) was used to root the trees and was aligned with *Parodon*'s sequences using ClustalW. Sequences were submitted to jModelTest 2 (Darriba *et al*., 2012) using corrected Akaike information criterion (AICc) to select the best-fit nucleotide evolution GTR + G model for downstream analyses.

Four methods of species delimitation were applied: (a) General mixed Yule-coalescent (GMYC) (Pons *et al*., 2006; Fujisawa, Barraclough, 2013); (b) Bayesian Poisson Tree Processes (bPTP) (Zhang *et al*., 2013); (c) Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al*., 2012); (d) Assemble Species by Automatic Partitioning (ASAP) (Puillandre *et al*., 2021). For GMYC analysis, an ultrametric gene tree was inferred in Beast v. 2.6.1 (Bouckaert *et al*., 2019). The GTR + G substitution model, a Strick Clock and the Yule model *prior* were employed. The Markov chains included 100,000,000 generations, storing trees every 10,000 generations, to obtain a total of 10,001 trees. Tracer v. 1.6 (Rambaut *et al*., 2014) was used to examine the average standard deviation of split frequencies and the convergence of MCMC searches, with >200 considered an appropriate effective sample size. The first 1,000 trees were discarded as burn-in, and 9,001 trees were summarized in the Maximum Clade Credibility tree (MCC) from the posterior distribution in TreeAnnotator v. 2.6 (Bouckaert *et al*., 2019). The tree was imported into the R software (R Development Core Team, 2013), and a species delimitation test was made with the SPLITS package (Species' Limits by Threshold Statistics; http://r-forge.r-project.org/projects/splits/), using the single threshold

method. For bPTP analysis, a Bayesian inference tree was generated in MrBayes 3.2 program (Huelsenbeck, Ronquist, 2001) applying 100,000,000 iterations of MCMC, sampling trees every 10,000 generations and burn-in of 10,000,000, and used as an input file in the bPTP web server (https://species.h-its.org/ptp/). 500,000 MCMC generations run (thinning = 500), and the other parameters were kept at their default values. The alignment generated for the sequences was used as an input file for the delimitation of species in the ABGD web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) and in the ASAP web server (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html), the Kimura (K80) TS/TV was selected for distance mode, assuming that Ts and TV have different rates, and the others parameters were kept at default, as suggested by Puillandre *et al.* (2012, 2021).

RESULTS

Cytogenetic data. The 2n of 54 was verified for *Parodon* sp. and *P.* cf. *buckleyi*, but with distinct karyotypic formula: 48 m/sm + 6 st and FN = 108 for *Parodon* sp. (Figs. 2A–B), and 50 m/sm + 4 st and FN = 108 for *P.* cf. *buckleyi* (Figs. 2E–F) (Tab. 1). Heterochromatin was preferentially located in the centromeric and terminal portions of the chromosomes in *Parodon* sp. and *P.* cf. *buckleyi* (Figs. 2C–D, G–H). Interstitial heterochromatic blocks were located at the q arms of pairs 6, 13, 26, and 27 in *Parodon* sp. (Figs. 2C–D) and at the q arms of pairs 8, 13, and 27 in *P.* cf. *buckleyi* (Figs. 2G–H).

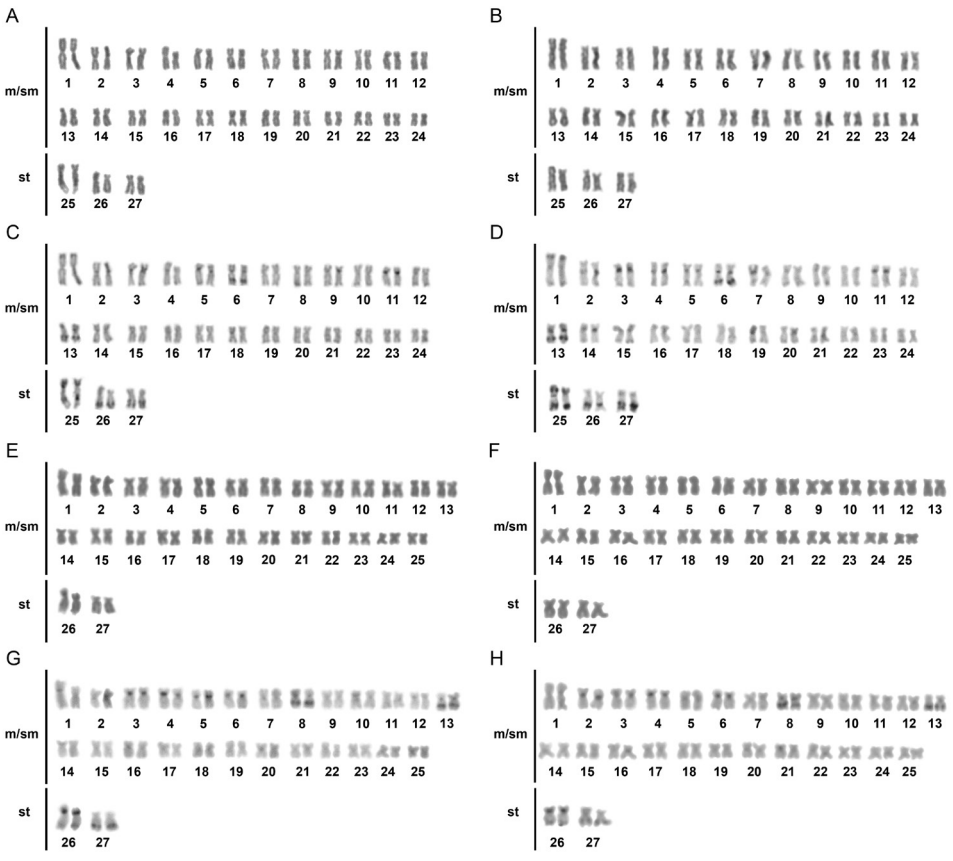

FIGURE 2 | Karyotypes of *Parodon* sp. (**A–D**) and *Parodon* cf. *buckleyi* (**E–H**) after conventional Giemsa-staining (**A–B**, **E–F**) and C-banding (**C–D**, **G–H**). **A**, **C**, **E** and **G** correspond to male individuals, and **B**, **D**, **F** and **H** correspond to female individuals. Scale bar = 10µm.

TABLE 1 | Cytogenetic and chromosomal markers data available for *Parodon* species. ♂: male; ♀: female; m: metacentric; sm: submetacentric; st: subtelocentric; p: short arm; q: long arm; Ref.: References. 1 – Moreira-Filho *et al.* (1993); 2 – Jesus, Moreira-Filho (2000); 3 – Moreira-Filho *et al.* (1984); 4 – Moreira-Filho *et al.* (1985); 5 – Vicente *et al.* (2001); 6 – Vicente *et al.* (2003); 7 – Centofante *et al.* (2002); 8 – Bellafronte *et al.* (2005); 9 – Bellafronte *et al.* (2011); 10 – Schemberger *et al.* (2011); PS: Present study.

Species	Karyotypic formulae	Sex chromosomes system	18S rDNA sites	5S rDNA sites	pPh2004 sites	Ref.
Parodon hilarii	54 m/sm ♂ 53 m/sm + 1 st ♀	ZZ/ZW	16q m (terminal)	11p m (proximal)	2pq m, 4p m, 9q m, 15pq sm, 21p m, 25q m, Zq sm, and Wp sm (all sites terminals)	1, 2, 5, 6, 10
Parodon moreirai	54 m/sm ♂♀	ZZ/ZW	15q m (terminal)	9p m (proximal)	9q m, Zq sm, and Wp m (all sites terminals)	7, 10
Parodon nasus	48 m/sm + 6 st ♂♀	Proto-sex chromosome	25q st (terminal)	25p st (proximal)	6q sm (terminal and interstitial), and 13q sm, 26q st and 27q st (terminals)	2, 3, 4, 5, 7, 8, 9, 10
Parodon pongoensis	50 m/sm + 4 st ♂♀	Proto-sex chromosome	2q m (terminal)	9 m (proximal)	13q m (terminal)	2, 5, 9, 10
Parodon sp.	48 m/sm + 6 st ♂♀	Proto-sex chromosome	25q st (terminal)	25p st (proximal)	6q sm, 26q st, and 27q (all sites terminals)	PS
Parodon cf. *buckleyi*	50 m/sm + 4 st ♂♀	Proto-sex chromosome	26q st (terminal)	26p st (proximal)	8 sm (centromeric and q terminal) and 27q st (terminal)	PS

Double-FISH using 18S rDNA and 5S rDNA probes showed a syntenic arrangement in *Parodon* sp. and *P.* cf. *buckleyi* karyotypes, with 45S rDNA located on the 25q terminal region, and 5S rDNA located on the 25p proximal region in *Parodon* sp. (Fig. 3A), and 45S rDNA located on the 26q terminal region, and 5S rDNA located on the 26p proximal region in *P.* cf. *buckleyi* (Fig. 3B). The pPh2004 satellite DNA was located on the q terminal regions of submetacentric pair 6, and subtelocentric pairs 26 and 27 in *Parodon* sp. (Fig. 3C). For *P.* cf. *buckleyi*, pPh2004 satellite DNA was located on the centromeric and q terminal region in the submetacentric pair 8, and on the q terminal region in the subtelocentric pair 27 (Fig. 3D).

(GATA)n showed an evident block at the p arm of a submetacentric chromosome pair (pair 9) and some dispersed signals in *Parodon* sp. chromosomes (Fig. 4A). In *P.* cf. *buckleyi*, evident signals of (GATA)n were located on the p arms of a submetacentric (pair 9) and a subtelocentric (pair 25) chromosome, with dispersed signals in other chromosomes (Fig. 4B). The *Tc1-Mariner* repetitive DNA was located on the terminal regions of almost all chromosomes in *Parodon* sp. and *P.* cf. *buckleyi* (Figs. 4C–D). *Helitron* sequence was located on the q distal regions in chromosome pair 6 in *Parodon* sp. (Fig. 4E), and in chromosome pairs 8 and 27 in *P.* cf. *buckleyi* (Fig. 4F).

Molecular analysis. Partial COI gene sequences were obtained for *Parodon* sp., *P.* cf. *buckleyi*, *P. hilarii*, and *P. pongoensis* (GenBank accession numbers: PV834389–PV834411). All sequences were of high quality and showed no evidence of indels, deletions, or stop codons. Sequences were aligned with the *Parodon* sequences retrieved from BOLD and GenBank, and a 602 bp matrix comprising 49 sequences with 167 polymorphic sites was generated. Twenty haplotypes were identified in the matrix:

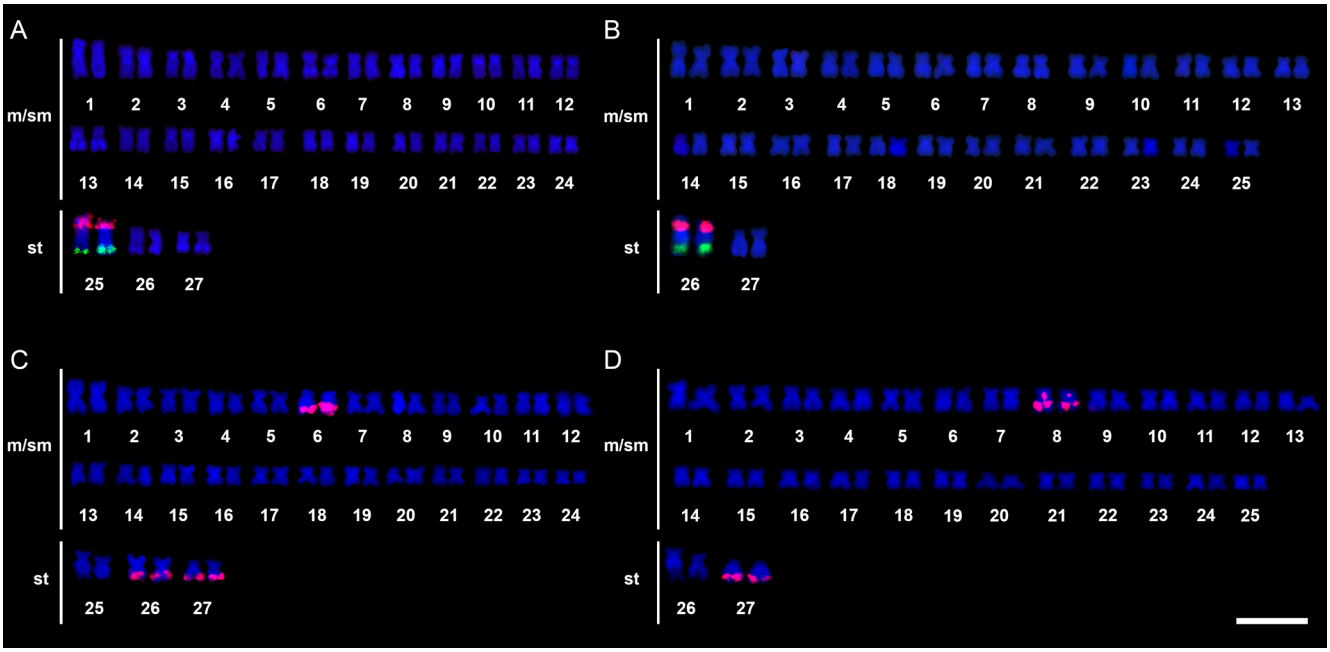

FIGURE 3 | Karyotypes of *Parodon* sp. (**A**, **C**) and *Parodon* cf. *buckleyi* (**B**, **D**) submitted to fluorescence *in situ* hybridization using 18S (green signals) and 5S (red signals) rDNA probes (**A**–**B**), and pPh2004 satellite DNA probe (**C**–**D**). Scale bar = 10 µm.

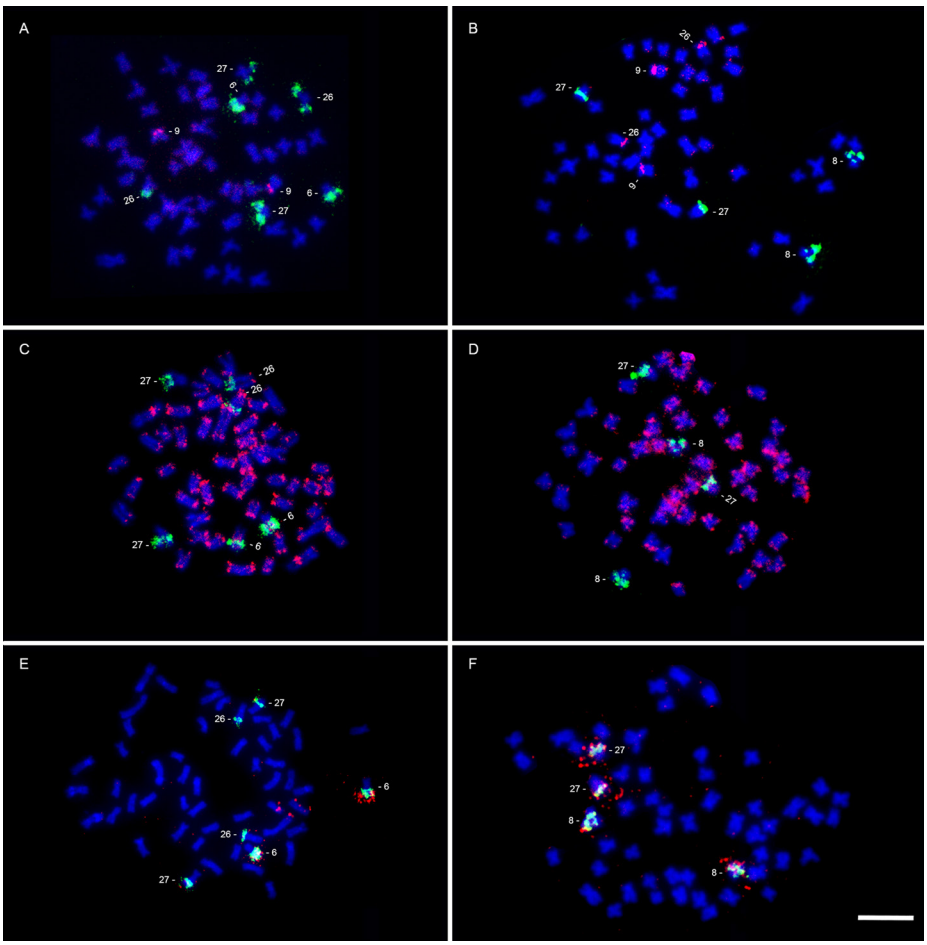

FIGURE 4 | *Parodon* sp. (**A**, **C**, **E**) and *Parodon* cf. *buckleyi* (**B**, **D**, **F**) metaphases submitted to double FISH using pPh2004 satellite DNA probe (green signals; **A**–**F**) and $(GATA)_n$ sequence (red signals; **A**–**B**), *Tc1-mariner* probe (red signals; **C**–**D**), and *Helitron* probe (red signals; **E**–**F**). Scale bar = 10 µm.

Parodon apolinari, *P. caliensis*, *P. guyanensis*, *P. hilarii*, and *P. suborbitalis* showed unique haplotypes, *P. nasus* presented two haplotypes, while four different haplotypes for each one of the populations of *Parodon* sp., *P.* cf. *buckleyi*, and *P. pongoensis* were observed (Fig. 5A). *Parodon alfonsoi* and *P. magdalenensis* shared a unique haplotype (Fig. 5A).

The intraspecific genetic distance ranged from 0 to 0.46% and the interspecific genetic distance ranged from 0 to 18.4% among the *Parodon* species (Fig. 5B; Tab. **S2**). The phylogenetic tree demonstrated monophyly for the nine Parodontidae species analyzed (Fig. 5C). The recovered topology showed the occurrence of eight groups: (1) *Parodon guyanensis*; (2) *P. pongoensis*; (3) *P. hilarii*; (4) *P. caliensis*; (5) *P. suborbitalis*; (6) *P. magdalenensis* + *P. alfonsoi*; (7) *P. apolinari*; (8) *P.* cf. *buckleyi*, *P. nasus* and *Parodon* sp. (Fig. 5C). Group 8 also indicated that *P.* cf. *buckleyi* is the sister group to *P. nasus*, with *Parodon* sp. forming a sister lineage to both.

The four molecular methods of species delimitation tested recovered eleven MOTUs (outgroup included): *Parodon apolinari*, *P.* cf. *buckleyi*, *P. caliensis*, *P. guyanensis*, *P. hilarii*, *P. magdalenensis* + *P. alfonsoi*, *P. nasus*, *P. pongoensis*, *Parodon* sp., *P. suborbitalis*, and *Leporinus piau* (Fig. 5C). The GMYC analysis suggested eight clusters (confidence interval 8–8) and the number of eleven entities (confidence interval 11–12; likelihood of null model: 316.5217; maximum likelihood of GMYC model: 339.2739; likelihood ratio: 45.50428; LR test: 1.314836e-10***; threshold time: -0.0058). The ML solution for bPTP also identified 11 species for the analyzed tree. Eight partitions were observed with the

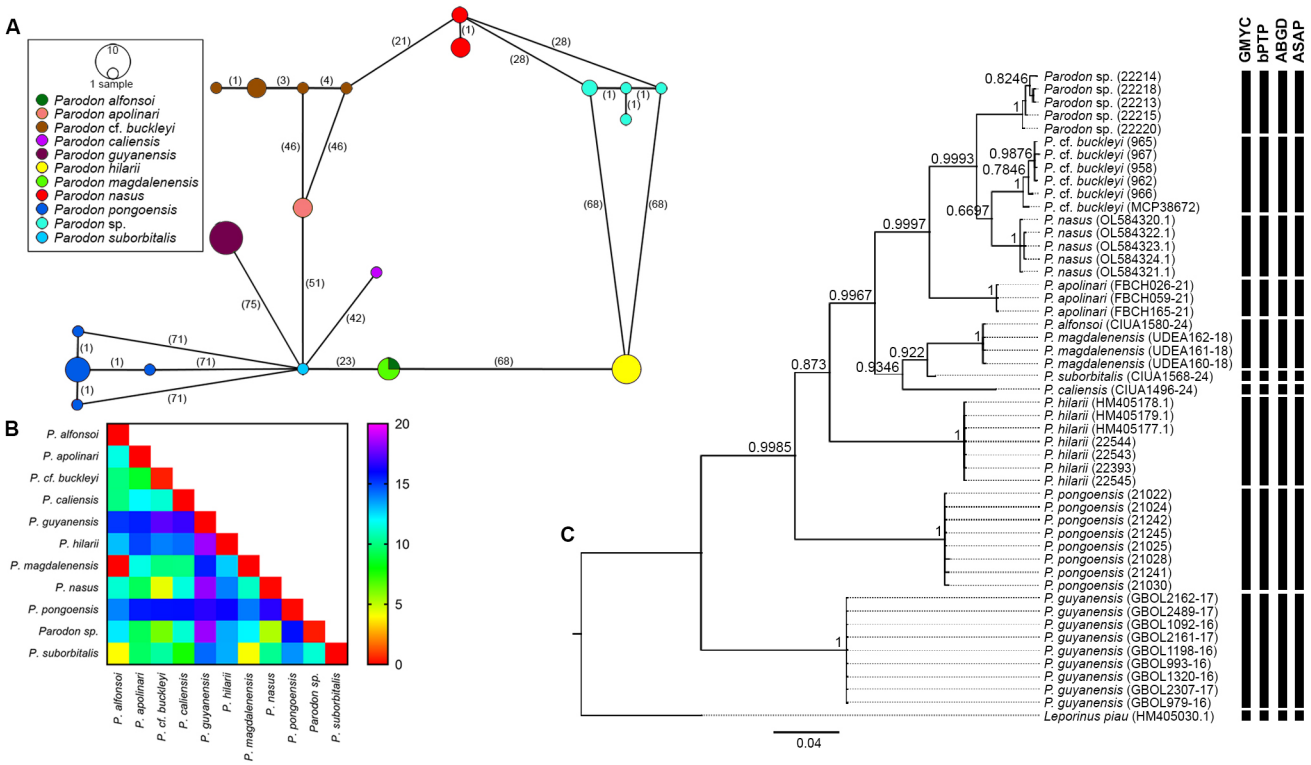

FIGURE 5 | Molecular data of *Parodon* species. **A.** Haplotype network of COI sequences. **B.** Heatmap of genetic distance among *Parodon* species (values were presented in %). **C.** Bayesian inference tree showing phylogenetic relationships and molecular species delimitation (vertical black bars) (Numbers on the branches correspond to posterior probability).

ABGD method: one partition found seventeen groups, while the other seven found eleven groups. For partition 5, eleven species (outgroup included) were observed (prior maximal distance P = 0.007743). The best partition identified by the ASAP method recognized eleven species (ASAP-score: 1.50; *p-value*: 8.93e-03; W: 2.51e-03; Threshold dist.: 0.022016; outgroup included).

DISCUSSION

The integration of multiple lines of evidence (morphology, cytogenetics, and DNA barcoding, among others) within a unified framework underpins the concept of integrative taxonomy (Dayrat, 2005). This approach, combining morphological, cytogenetic, and molecular data, has proven decisive for delimiting some Neotropical characiforms (Ramirez *et al*., 2017; Gavazzoni *et al*., 2024). Species in the "*P. suborbitalis* complex" lack reliable external diagnostic characters and have been grouped primarily by coloration, especially the shape of the lateral stripe, and geographic distribution (Pavanelli, 2003; Londoño-Burbano *et al*., 2011). Within this complex, species exhibit overlaps in morphological traits that typically serve as diagnostic characters for other Parodontid species, such as the number of pored scales along the lateral line, premaxillary cusps, and preanal scales (Pavanelli, 2003; Londoño-Burbano *et al*., 2011). Comprehensive morphological assessments of *Parodon* sp. and *P.* cf. *buckleyi* remain necessary to clarify their taxonomic placement. Notably, *Parodon* cf. *buckleyi* may also represent a distinct MOTU in *Parodon*, given that the type locality of *P. buckleyi* is in Ecuador, and populations from the Machado River sub-basin appear isolated (Pavanelli, 1999). In the absence of decisive external diagnostic, cytogenetic techniques (cytotaxonomy) and DNA barcoding have been effective tools for species delimitation in Parodontidae (Bellafronte *et al*., 2011, 2013; Schemberger *et al*., 2011; Azambuja *et al*., 2023).

Parodon sp. and *P.* cf. *buckleyi* show a karyotype quite similar, with the same 2n and FN, and no heteromorphic sex chromosomes, indicating a close relationship between these lineages. Nevertheless, differences were identified in the karyotype formula, heterochromatin accumulation, and the *in situ* localization of repetitive DNAs. Within *Parodon*, cytogenetic studies have been limited to *P. hilarii*, *P. moreirai*, *P. nasus*, and *P. pongoensis*. All these species show 2n = 54 and FN = 108, however, heteromorphic sex chromosomes (ZZ/ZW) have been described for *P. hilarii* and *P. moreirai*, while a pair of proto-sex chromosomes has been proposed for *P. nasus* and *P. pongoensis* (Tab. 1). The cytogenetic analysis reveals that *Parodon* sp. and *P.* cf. *buckleyi* have chromosomal features similar to *P. nasus* (Tab. 1). The syntenic condition for 45S and 5S rDNAs (*i.e*., 45S rDNA located on the q arm and 5S rDNA located on the p arm of the first subtelocentric chromosome pair) observed in *Parodon* sp. and *P.* cf. *buckleyi* has been proposed as an apomorphic chromosomal condition in *P. nasus* (Bellafronte *et al*., 2005, 2011; Azambuja *et al*., 2022a). Thus, the homeology of the rDNA-bearing chromosome pair designates this condition as a synapomorphy shared by *Parodon* sp., *P. nasus*, and *P.* cf. *buckleyi* within Parodontidae, and differentiates these species from *P. hilarii*, *P. moreirai*, and *P. pongoensis*.

The number and chromosomal locations of the pPh2004 satellite DNA sites are other chromosomal characteristics that differ among Parodontidae karyotypes (Bellafronte

et al., 2011; Schemberger *et al*., 2011). However, some conservation is observed in homeologous chromosomal loci of closely related species, which is useful for karyotype-based species delimitation (Schemberger *et al*., 2011; Traldi *et al*., 2020). Differences in the distribution of pPh2004 satellite DNA are evident between *Parodon* sp. and *P.* cf. *buckleyi*, suggesting interspecific divergence between these taxa. Furthermore, both the number and location of pPh2004 satellite DNA sites show interspecific divergence when compared to other *Parodon* species such as *P. pongoensis*, *P. nasus*, *P. moreirai*, and *P. hilarii* (Tab. 1). Satellite DNAs loci have been considered hotspots for chromosomal rearrangements in many karyotypes due to their dynamic and fast-evolving nature (Paço *et al*., 2013; Gatto *et al*., 2018; Moraes *et al*., 2023; Ribas *et al*., 2026). The pPh2004 site, observed in the centromeric region of chromosome pair 8 in *P.* cf. *buckleyi*, represents a species-specific marker within *Parodon* species, which may have resulted from ectopic recombination or a chromosomal inversion involving pPh2004 sequences.

Chromosome painting with the W heterochromatic fraction of *Apareiodon* sp. (W*Ap* probe) in combination with the pPh2004 satellite DNA was employed to propose the proto-sex chromosome pair in *P. nasus* and *P. pongoensis* (Schemberger *et al*., 2011). Subsequently, the proto-sex chromosome of *P. nasus* and *P. pongoensis* was also described as containing (GATA)n repeats in the heterochromatic region of the short arm (Ziemniczak *et al*., 2014). Currently, it is known that the W heterochromatic region of *Apareiodon* sp. is predominantly composed of microsatellite expansion and DNA transposons, especially *Helitron*, *Tc1-Mariner*, and *EnSpm* (Schemberger *et al*., 2019; Oliveira *et al*., 2025). The *in situ* localization shows that chromosome pairs 6 and 8 are enriched with these repetitive elements in *Parodon* sp. and *P.* cf. *buckleyi*, respectively, supporting the proposition that they represent the proto-sex chromosomes. Besides that, the colocalization of pPh2004 sites with the *Helitron* DNA transposon suggest that this transposable element may mediate satellite dispersal, promoting karyotype diversification between *Parodon* sp. and *P.* cf. *buckleyi*.

In addition to cytogenetic data, the K2P genetic distances and molecular methods of species delimitation indicate that *Parodon* sp. and *P.* cf. *buckleyi* represent two distinct MOTUs within Parodontidae. Ward (2009) suggested a K2P value of 2% as a threshold for fish species identification, a criterion supported by Pereira *et al*. (2013) for various Neotropical fish. Within Parodontidae, interspecific K2P values above 2% have been reported (Bellafronte *et al*., 2013; Santos *et al*., 2018; Traldi *et al*., 2020). However, low genetic divergence based on COI has been reported between some paired species (*Apareiodon piracicabae* (Eigenmann, 1907) and *A. vittatus* Garavello, 1977, and between *A. machrisi* Travassos, 1957, and *A. cavalcante* Pavanelli & Britski, 2003), with K2P distances below 2% (Bellafronte *et al*., 2013; Traldi *et al*., 2020). Despite this reduced mitochondrial divergence, cytogenetic and morphological differences support the validity of these taxa as distinct species, suggesting that they may represent cases of recent or ongoing speciation (Bellafronte *et al*., 2013; Traldi *et al*., 2020). Haplotype network, K2P distance, and phylogenetic analyses suggest a close evolutionary relationship among *Parodon* sp., *P. nasus*, and *P.* cf. *buckleyi*. Cytogenetic comparisons provide additional support for the molecular findings and suggest recent divergence among these species. The quite similar karyotypic formula, the synteny of 18S and 5S rDNAs, and the presence of a pair of proto-sex chromosomes reaffirm their close relationship and distinguish these species karyotypically from other congeners.

The molecular divergence of COI sequence analyzed here comprises several well-supported *Parodon* lineages exhibiting high interspecific K2P distances (Tab. **S2**; Fig. 5). When the molecular phylogeny is compared with the geographic distribution of species across South American river basins (Fig. 1), a clear biogeographic pattern emerges, in which species occurring within the same sub-basin show closer phylogenetic relationships to one another. Most of the species in the previously described "*P. suborbitalis* complex" inhabit regions close to the Andean Cordillera (Londoño-Burbano *et al*., 2011). However, the presence of multiple well-supported branches representing *Parodon* species, together with the high observed genetic distances, indicates that the "*P. suborbitalis* complex" is not monophyletic.

The establishment of major Neotropical drainage systems, including the Amazon, Orinoco, and La Plata, occurred over the past 10 million years (Myr), highlighting the influence of Andean foreland dynamics and paleoarches on present-day watersheds through vicariance and headwater-capture events (Montoya-Burgos *et al*., 2003; Hubert, Renno, 2006). Within the proposed "*P. suborbitalis* complex", only *P. alfonsoi*, *P. magdalenensis*, and *P. suborbitalis* exhibit a close phylogenetic relationship, all occurring in the northern portion of the trans-Andean region. However, these data should be interpreted with caution, as *P. alfonsoi* and *P. magdalenensis* share a haplotype and exhibit a K2P distance of 0%, suggesting recent diversification, incomplete lineage sorting, or potential hybridization, given that both species occur in the Magdalena River basin. Also inhabiting the northern trans-Andean region, *P. apolinari* and *P. caliensis*, originally not included in the "*P. suborbitalis* complex" due to morphological features (Pavanelli, 1999), show well-supported subclades close to *P. suborbitalis* in the molecular phylogeny, corroborating the non-monophyly of the species complex. These findings suggest that multiple *Parodon* lineages may have undergone diversification in the trans-Andean region. It is important to note that, of the nine species originally included in the complex (Londoño-Burbano *et al*., 2011), three of them, *P. bifasciatus*, *P. carrikeri*, and *P. atratoensis*, were not included in the present analyses due to the absence of available data, which limits a comprehensive assessment of the monophyly of the originally proposed complex.

All other *Parodon* species analyzed are cis-Andean and, similarly, have branches in the phylogenetic tree consistent with a biogeographic context. *Parodon guyanensis* occurs in the freshwater systems of the Guianas region, shows high genetic distance from congeners, and has a well-supported phylogenetic branch. Similarly, *P. pongoensis* and *P. hilarii*, which occur in the freshwater systems of the Araguaia-Tocantins and São Francisco, respectively, also have well-supported phylogenetic branches. Finally, *Parodon* sp., *P.* cf. *buckleyi*, and *P. nasus*, which are grouped in a single phylogenetic clade, inhabit the Amazon (first two) and La Plata (last one) basins. Marine regressions and changes in the Andean forelands between 10 and 8 Myr ago played a key role in the final establishment of the Amazon Basin (Hubert, Renno, 2006). In addition, around 10 Myr ago, the Paraná–Paraguay system (La Plata basin) separated from the proto-Amazon (Lundberg *et al*., 1998). Nevertheless, some evidence suggests that headwater-capture events connecting the Paraná system to the Amazon occurred within this timeframe (Räsänen *et al*., 1995; Lundberg *et al*., 1998), which could explain the dispersion and similarity among *Parodon* sp., *P.* cf. *buckleyi*, and *P. nasus*.

In this context, the *Parodon* species analyzed here are arranged in smaller groups presenting consistent genetic distances, which are closely associated within a biogeographic framework. These findings in *Parodon* are consistent with other phylogenetic studies of Neotropical freshwater fishes, which demonstrate that species diversification is closely associated with the geological history of South America's hydrographic basins (Ribolli *et al*., 2021; Elías *et al*., 2023; Volpi *et al*., 2023).

It is important to emphasize that the molecular phylogeny presented here is based on the substitution patterns of a single mitochondrial gene. Although mitochondrial markers are informative and widely used in fishes (Morais-Silva *et al*., 2023; Souza *et al*., 2023; Almeida *et al*., 2024, among others), multilocus approaches incorporating independent nuclear loci provide a more robust reconstruction of species trees by better accounting for processes such as incomplete lineage sorting, species tree discordance, and historical gene flow (López-Fernández *et al*., 2010; Frable *et al*., 2016; de Queiroz *et al*., 2020; Ramirez *et al*., 2020). More recently, phylogenomic frameworks based on genome-wide data, such as ultraconserved elements (UCEs), RADseq, among others, have substantially improved phylogenetic resolution and provided greater power to infer diversification processes, timing of lineage splits, and patterns of speciation (Díaz-Arce *et al*., 2016; Ilves *et al*., 2018; Souza *et al*., 2022; Wang *et al*., 2026). Therefore, future studies integrating multilocus or phylogenomic datasets will be essential to refine evolutionary relationships and further test species boundaries within the *Parodon*. Despite the limitation of single-locus analyses, cytogenetic data indicate low divergence among *Parodon* sp., *P.* cf. *buckleyi*, and *P. nasus*, but clearly distinguish *P. moreirai*, *P. pongoensis*, and *P. hilarii* from both these species and from each other.

In conclusion, this study analyzed two *Parodon* species from the Amazon basin and identified several cytogenetic and molecular characteristics that differentiate them from other *Parodon* species. The findings support the hypothesis that *Parodon* sp. may constitute an undescribed species. Additionally, the presence of *P.* cf. *buckleyi* in the Madeira River basin, which is distant from its type locality in western Amazonia, Ecuador, may also reflect the existence of another undescribed species within the genus *Parodon*. Molecular analysis of specimens from the type locality of *P. buckleyi* would help clarify its taxonomic status. Furthermore, the data indicate that the "*Parodon suborbitalis* complex" comprises distinct lineages that are closely associated within a biogeographic framework.

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ETHICAL STATEMENT

Fish were collected with the authorization of the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO), Sistema de Autorização e Informação em Biodiversidade (SISBIO, license numbers 10538 and 15117), and Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN Nº AE12D3D). The procedures were approved by the Comição de Ética no Uso de Animal of the Universidade Estadual de Ponta Grossa (Process CEUA Nº 6/2019) and Biosafety Certification according to Comissão Técnica Nacional de Biossegurança – CTNBio (CQB Nº 0063/98). The procedures of this study are in agreement with the Ethics Committee of Animal Usage of the Universidade Estadual de Ponta Grossa, Brazil (Protocol: 06/2019).

DATA AVAILABILITY STATEMENT

All the data supporting the findings are included in this published article, its supplementary information files.

AI STATEMENT

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The authors declare no competing interests.

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