Cytogenetics and DNA barcode reveal an undescribed *Apareiodon* species (Characiformes: Parodontidae)

Emanoel Oliveira dos Santos¹, Geize Aparecida Deon², Rafael Bonfim de Almeida¹, Ezequiel Aguiar de Oliveira³, Viviane Nogaroto², Hugmar Pains da Silva⁴, Carla Simone Pavanelli⁶, Marta Margarete Cestari¹, Luiz Antonio Carlos Bertollo³, Orlando Moreira-Filho⁵ and Marcelo Ricardo Vicari¹²

¹Departamento de Genética, Programa de Pós-Graduação em Genética, Universidade Federal do Paraná, Curitiba, PR, Brazil.
²Departamento de Biologia Estrutural, Molecular e Genética, Universidade Estadual de Ponta Grossa, Ponta Grossa, PR, Brazil.
³Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos, São Paulo, SP, Brazil.
⁴Laboratório de Citogenética e Genética Animal, Instituto de Biociências, Universidade Federal de Mato Grosso, Cuiabá, MT, Brazil.
⁵Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupélia), Universidade Estadual de Maringá, Maringá, PR, Brazil.

Abstract

Parodontidae is a small group of fish and some species are particularly difficult to identify due to the lack of sufficiently consistent morphological traits. Cytogenetically, the species possess 2n = 54 chromosomes and are either sex-homomorphic or sex-heteromorphic (regarding its chromosomes). We evaluated data on color, tooth morphology, cytogenetics, and mitochondrial markers (COI) in *Apareiodon* specimens from the Aripuanã River (Amazon basin) and the results were compared to other congeneric taxa. Morphological results show an overlap of body color and tooth morphology to other known *Apareiodon*. The cytogenetics data showed that the 2n = 54 chromosomes, 50 m/sm + 4 st and, a ZZ/ZW sex chromosome system in *Apareiodon* sp. are common to other species of the genus. However, the number and chromosomal localization of the 45S ribosomal and pPh2004 satellite DNA sites, in addition to W chromosome localization of the pPh2004 appear to be exclusive cytogenetic features in *Apareiodon* sp. Our phylogenetic tree revealed well-supported clades and confirmed, by barcode species delimitation analysis, a new Molecular Operational Taxonomic Unit (MOTU) for *Apareiodon* sp. (Aripuanã River). As a whole, the above features support the occurrence of a new species of the *Apareiodon*, thus far unknown for the Parodontidae.

Keywords: Chromosomal differentiation, hidden diversity, repetitive DNA, sex chromosomes.

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Introduction

The family Parodontidae is currently composed of three genera: *Apareiodon* Eigenmann, 1916; *Parodon* Vaillanciennes, 1849 and *Saccodon* Kner, 1863 (Pavanelli, 2003), with 32 valid species (Eschmeyer and Fong, 2017). Its species can be differentiated from the other Characiformes by the following combined features: an edentulous lower jaw in the anterior region and spatulate mandible, pedunculated and multicuspid premaxillary teeth with wide distal border distributed in a single series, and by the absence of upper lip and fontanelle (Pavanelli, 2003). The genera are mainly characterized by variation in the number of undivided rays in the pectoral fins. However, the color pattern with one regular black longitudinal stripe or several vertical bands, in addition to the shape and number of the tooth cusps adjacent to the premaxillary symphysial tooth, can also be used in the species identification (Pavanelli, 2003).

Cytogenetic analyzes in Parodontidae revealed a conserved diploid number of 54 chromosomes, most of them meta/submetacentric, with few or none subtelocentric chromosome (Moreira-Filho *et al.*, 1980; Jesus and Moreira-Filho, 2000; Rosa *et al.*, 2006; Vicari *et al.*, 2006). Acrocentric chromosomes are an exception, only found in *A. affinis* of the lower Paraná River system (Jorge and
Moreira-Filho, 2000, 2004; do Nascimento et al., 2018). Differences in karyotypes, such as distinct karyotypic formulas, number and localization of the satellite DNA pPh/2004 and 18S and 5S rDNA sites, distribution of the heterochromatic bands and occurrence of sex chromosomes (Moreira-Filho et al., 1984, 1985; Bellafronte et al., 2011, 2012; Schemberger et al., 2011, 2014, 2016; Ziemniczak et al., 2014) appear as exclusive derived species features.

However, one of the most outstanding cytogenetic characteristics in the differentiation of Parodontidae species is the occurrence of sex chromosome systems. This way, besides species without heteromorphic sex chromosomes and with proto-sex ones, heteromorphic ZZ/ZW sex chromosomes and multiple sex systems of the ZZ/ZW1W2 type also occur (Schemberger et al., 2011). Heterochromatization is commonly associated to the morphological differentiation of the W chromosome (Centofante et al., 2002; Vicente et al., 2003; Vicari et al., 2006; Schemberger et al., 2011; Bellafronte et al., 2012). Accumulation of satellite sequences and transposable elements (TEs) were events responsible for molecular differentiation and erosion of the W chromosome gene activity, allowing the identification of pseudo-autosomal (PAR) and W specific (WSR) regions in Parodontidae (Schemberger et al., 2014; Ziemniczak et al., 2014). In addition, repetitive sequences also promoted the differentiation among autosomes, acting on the karyotypic diversification of this group (Bellafronte et al., 2011; Schemberger et al., 2011, 2014, 2016; Ziemniczak et al., 2014; Traldi et al., 2016; do Nascimento et al., 2018).

Integrative cytogenetic and DNA barcoding studies were effective in detecting chromosome evolution and species richness within supposedly homogeneous taxa of A. affinis (do Nascimento et al., 2018). Indeed, molecular analysis contributes to reveal a hidden biodiversity (Cunha et al., 2017; Ramirez et al., 2017; do Nascimento et al., 2018) and has been widely used for identification and delimitation of neotropical fish species (Carvalho et al., 2011; Pereira et al., 2011, 2013; Ramirez and Galetti Jr, 2015; Machado et al., 2016). In this sense, one of the most used genes for species identification is the mitochondrial cytochrome c oxidase subunit 1 (COI) gene, which was proposed by Hebert et al. (2003) as a DNA barcode methodology. Thus, DNA barcoding and population genetics can be used to define discrete genetic lineages, characterizing Molecular Operational Taxonomic Units (MOTUs) and/or revealing reciprocal monophony (Floyd et al., 2002).

In this study, morphological patterns, chromosomal markers, and DNA barcode analysis were used with the objective of identifying a probable undescribed Apareiodon species from the Aripuanã River, Mato Grosso State, Brazil, here named as Apareiodon sp. In addition, we discuss the chromosomal evolution and species diversification within the Parodontidae.

Material and Methods

Biological samples

Chromosome studies were performed on 32 specimens (23 males and 9 females) of Apareiodon sp., collected in the Aripuanã River, Amazon basin (10°09’57.8"S and 59°26’54.9" W). The chromosomal and DNA material was obtained from the Laboratory of Fish Cytogenetics of the Universidade Federal de São Carlos. The procedures were in agreement with the Ethics Committee for Animal Use of the Universidade Estadual de Ponta Grossa (Protocol: 29/2016). The fish were fixed in 10% formalin and, after 48 hours, preserved in 70% ethanol. The specimens were identified based mainly on a black band on the third distal edge of the dorsal fin, but also on the combination of other morphological features, such as a black lateral band without up and downwards projections, two maxillary teeth, premaxillary teeth with a rounded cutting edge and 9 to 11 cusps, among others. Voucher specimens were deposited at the Coleção Ictiológica of Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupélia) of Universidade Estadual de Maringá (NUP 199988).

Analysis of body color, symphysial teeth, and tooth cusps

Body color analysis was performed according to the method described by Pavanelli and Bristki (2003). Photos of unfixed and fixed specimens were used in order to ascertain the presence of the black dorsal-fin band and yellowish tone in Apareiodon sp. (Aripuanã River). Also, an extraction and analysis of the symphysial teeth was performed following do Nascimento et al. (2018). Teeth were photographed using a bright-field microscope (Olympus BX43) coupled to a DP72 CCD camera (Olympus) at 40x magnification for further counting of the cusps number.

Chromosomal preparations

Metaphase chromosomes were obtained from kidney cells, after in vivo treatment with colchicine and conventional air-drying preparation (Bertollo et al., 2015), and the chromosomal preparations in slides were submitted to conventional Giemsa staining 5% in phosphate buffer (pH = 6.8). Constitutive heterochromatin was detected by the C-banding method (Sumner, 1972). The images were captured with a microscope (Olympus BX43) coupled to a DP72 CCD camera (Olympus), edited, and arranged into karyotypes using Adobe Photoshop software CC 2015. Homologous chromosomes were paired and arranged into metacentric-submetacentric (m/sm) and subtelocentric (st) groups, according to Levan et al. (1964). Two arms were considered for each one of such chromosome types to determine the fundamental number (FN).
Fluorescence in situ hybridization (FISH)

FISH was performed following Pinkel et al. (1986), and five types of probes were used to localize complementary sequences in the metaphase chromosomes of Apareiodon sp. The following two sequences were isolated by PCR from the total genome of Apareiodon sp., according to the respective authors: 5S rDNA (Barros et al., 2017), 18S rDNA (Gross et al., 2010). In addition, the Parodon hilarii satellite DNA probe named pPh 2004 (Vicente et al., 2003), the heterochromatric fraction of the W chromosome of Apareiodon sp., named WAp (Vicari et al., 2010), and a (GATA)n microsatellite probe (Traldi et al., 2013) were used.

The 18S rDNA and (GATA)n probes were labeled using digoxigenin-11-dUTP hapten (Jena Bioscience), while 5S rDNA, pPh 2004, and WAp were labeled using biotin-16-dUTP hapten (Roche Applied Science). A general FISH protocol was followed under a stringency condition of ~80% (2.5 ng/μL probe, 50% formamide, 2xSSC, 10% dextran sulfate, 42 °C for 16 h). Post-hybridization washes were done at high stringency (50% formamide at 42 °C for 20 min, 0.1xSSC at 60 °C for 15 min, and 4xSSC 0.05% Tween at room temperature for 10 min). Streptavidin Alexa Fluor 488 (Molecular Probes) and an anti-digoxigenin rhodamine fab fragment (Roche Applied Science) were used for probe detection. The chromosomes were stained with DAPI (0.2 μg/mL) present in Vectashield mounting medium (Vector) and analyzed under epifluorescence microscopy.

Molecular analysis

For genomic DNA extraction, liver samples were used following the CTAB (cetyltrimethylammonium bromide) method of MURRAY AND Thompson (1980). DNA samples were used to amplify the barcode region of the mitochondrial gene cytochrome C oxidase subunit I (COI) by PCR. Amplification of the COI sequence was performed using the primers Fish F1 and Fish RI (Ward et al., 2005). The reaction mix was composed by 1x Taq Reaction buffer (200 mM Tris pH 8.4, 500 mM KCl), 40 μM dNTPs, 2 mM MgCl2, 2 U of Taq DNA polymerase (Invitrogen, Carlsbad, USA), 0.2 μM of each primer, and 100 ng of DNA template. The following reaction program was used: initial denaturation for 2 min at 94 °C, 35 cycles of 94 °C for 1 min, 52 °C for 40 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. After amplification, the samples were purified and submitted to nucleotide sequencing using an ABI-Prism 3500 Genetic Analyzer (Applied Biosystems).

COI sequences obtained for Apareiodon sp. from the Aripuanã River and for A. vittatus (Jangada River) were deposited in GeneBank (access numbers: MG827218 - MG827229). Additionally, COI sequences of A. piracicabae (Passa Cinco River), A. affinis (Cuiabã River), and A. affinis (Passa Cinco River) described by Bellafronte et al. (2013) were used. All sequences were first analyzed with Geneious 8.1.9 software (Kearse et al., 2012) and aligned using ClustalW algorithm, where possible sequencing errors were checked using the BLAST tool against the data deposited in GenBank (NCBI).

Estimates of genetic distances were obtained using the MEGA software 7.0.14 (Kumar et al., 2016) under the Kimura-2-Parameters (K2P) evolution model (Kimura, 1980). Gene flow, haplotype (h), and nucleotide (π) diversity analyses were performed by DnaSP software (Librado and Rozas, 2009). Population structuring and analysis of molecular variance (AMOVA) (Excoffier et al., 1992), were performed by the Arlequin 3.5.2.2 software (Excoffier and Lischer, 2010). The PopArt 1.7 program was used to generate a haplotype network through the median joining criterion (Leigh and Bryant, 2015). Structural analysis was performed by assignments of each individual to the respective populations using Bayesian inference in Structure 2.3.4 software (Pritchard et al., 2000) and Bayesian Analysis of Population Structure - BAPS 6 (Corander et al., 2004; Corander and Marttinen, 2006). The Bayesian inference tree was generated with the MrBayes 3.2 program (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003).

Results

Color and teeth

The Apareiodon sp. individuals showed a regular black longitudinal stripe extending from head to tail. The fin had a yellowish tone (pectoral, dorsal, caudal) and the end of the black stripe (Figure 1a). The symphysial teeth showed rounded edges, bearing 10 to 11 cusps (Figure 1b).

Cytogenetics

All specimens had 2n=54 chromosomes, FN=108, with 50 m/sm + 4st for both sexes. However, the 13th pair appeared heteromorphic in females, revealing a ZZ/ZW sex chromosome system (Figure 2). Both Z and W chromosomes were metacentric in form, but the W one was much larger, corresponding to the second largest chromosome of the complement (Figure 2). The heterochromatin bands were mainly pericentromeric, in addition to some terminal bands (Figure 2b, d). While the Z chromosome presented only a small proximal heterochromatric band in the short arm, the W chromosome had most of its long arm heterochromatric (Figure 2b, d).

FISH analysis with the 5S rDNA probe showed that this multigene family is localized in the p arm of pair 6 (Figure 3a). The in situ localization of 18S rDNA evidenced ten terminal clusters in pairs 2, 5, 9, 26 and 27, all in the long arms with exception of the pair 26 (Figure 3b). The DNA pPh2004 satellite was clustered in the terminal region of pairs 3, 5, 6 and, 11, in addition to ZW sex chromosomes, as well as the GATAn sequence although not in the same
chromosome pairs neither in the Z and W chromosomes (Figure 3c). In addition, the heterochromatic W Ap probe was also localized in the proximal region of the q arm of the Z chromosome and in a great extent of the q arm of the W chromosome (Figure 3d).

Molecular analysis

Twenty-six fragments of the COI gene from different Apareiodon individuals were analyzed. The sequences did not show insertions, deletions, stop codons, or sequencing errors. The sequences contained 706 nucleotides, with 183 variable sites, and a nucleotide diversity of $\pi = 0.13475$. The haplotype number considered was 16, with a haplotype diversity of $h = 0.93231$.

Genetic K2P divergence among species ranged from 5.9 to 23.4% and, when considering only Apareiodon sp. (Aripuanã River) in comparison to the other Apareiodon species, the K2P genetic distance was 20.6 to 23.4% (Table 1). The maximum likelihood tree and Bayesian analysis considering the substitution TIM2+G model (Posada, 2003) given by Jmodeltest revealed five main consistent branches: Apareiodon sp. (Aripuanã River), A. vittatus, A. piracicabae, A. affinis (Cuiabá River), A. affinis (Passa Cinco River) (Figure 4). Leporinus piou was used as out-group in the tree and demonstrated the ancestral relationship with all species analyzed in-group.

The barcode sequences of the Apareiodon species analyzed were also used for the haplotype network construction. This network showed that there is no haplotype overlap among the species, and that the haplotypes of same species are more closely related to each other when compared with others (Figure 5a). AMOVA analysis returned values of $\Phi_{ST} = 0.69829$. Regarding populational structuration inference, the analysis generated by BAPS indicated that there is no sequence overlap, thus evidencing independent groups (Figure 5b).

Discussion

Parodontids are mostly rheophilic, and therefore absent in the lower regions of the Amazon basin. However, the Apareiodon species from the upland rivers of the Amazon basin are morphologically less similar to Apareiodon sp. from the Aripuanã River than to congeners from the La Plata basin. In the body, the wide longitudinal stripe of Apareiodon sp. showed relatively similar patterns to Apareiodon species from the Paraná-Paraguai system (A. persimilis) and some congeners from the Amazon basin (A. affinis, A. piracicabae). The karyotypes of the males and females Apareiodon sp. specimens showed 11+11 sex chromosomes, with the ZZ sex chromosomes in the males and ZZW sex chromosomes in the females (Figure 2). The karyotypes were also characterized by the presence of symphesial teeth, which show the number of cusps in each tooth (Figure 3c).
Figure 3 - Karyotypes of the *Apareiodon* sp. submitted to probing for *in situ* localization: in (a) 5S rDNA (green); (b) 18S rDNA (red); (c) pPh 2004 (green) and (GATA)n (red) and; (d) WAp probe (green). Bar = 10 \( \mu \)m.

Figure 4 - Species tree showing phylogenetic relationships from the five analyzed species/populations of Parodontidae. To the left, topology for Maximum Likelihood (numbers on the branches are bootstrap values). To the right, Bayesian tree (numbers on the branches are posterior probability). The scale bar indicates nucleotide substitutions per site.

Table 1 - Estimates of evolutionary divergence over sequence pairs among groups.

| Species                        | *Apareiodon* sp. (Aripuanã) | *A. vittatus* | *A. affinis* (Cuiabá) | *A. affinis* (Passa Cinco) | *A. piracicabae* |
|--------------------------------|------------------------------|---------------|------------------------|----------------------------|-----------------|
| *Apareiodon* sp. (Aripuanã)    |                               |               |                        |                            |                 |
| *Apareiodon vittatus*          | 0.217 (±0.02)                |               |                        |                            |                 |
| *Apareiodon affinis* (Cuiabá)  | 0.215 (±0.02)                | 0.228 (±0.02) |                        |                            |                 |
| *Apareiodon affinis* (Passa Cinco) | 0.205 (±0.02)              | 0.206 (±0.02) | 0.059 (±0.01)          |                            |                 |
| *Apareiodon piracicabae*       | 0.233 (±0.02)                | 0.151 (±0.01) | 0.098 (±0.01)          | 0.077 (±0.01)              |                 |
| Intraspécific divergence       | 0.042 (±0.01)                | 0.033 (±0.00) | 0.00 (±0.00)           | 0.000 (±0.00)              | 0.000 (±0.00)   |

The number of base substitutions per site from averaging overall sequence pairs between groups are shown. Kimura-2-Parameters genetic distance mean and standard error. Analyses were conducted using the Kimura 2-parameter model. The analysis involved 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There was a total of 441 positions in the final dataset.
The 18S rDNA sites occupies the long arm terminal region of a single large subtelocentric chromosome pair in most species of Apareiodon (Moreira-Filho et al., 1984, 1985; Rosa et al., 2006; Vicari et al., 2006). Even though there are some few exceptions for multiple 18S rDNA sites occurring in Apareiodon (Bellafronte et al., 2009; Traldi et al., 2016), there is no case with such a high number as in Apareiodon sp., which may be related to some distinct chromosomal rearrangements, such as inversions, translocations and transposon-mediated transpositions (Symonová et al., 2013; Barbosa et al., 2017). On the other hand, the SS rDNA localization is a conserved trait in the Parodontidae, where the chromosome pair bearing these sequences appears as homologue in most species (Bellafronte et al., 2009, 2011). Additional 5S rDNA sites are a rare occurrence, and only Parodon nasus and A. affinis showed this site at a different chromosome and position (Bellafronte et al., 2009; Traldi et al., 2016; do Nascimento et al., 2018).

It was proposed that the differentiation of the ZZ/ZW sex system in Parodonidae is related to a paracentric inversion, in which the terminal WAp repetitive sequence was transposed to the proximal region of the short arm of a metacentric pair, with subsequent amplification leading to the differentiation of the W chromosome (Centofante et al., 2002; Vicente et al., 2003; Schemberger et al., 2011). Apparently, only Apareiodon hasemani presents a distinct stage of heterochromatinization on the p arm (Bellafronte et al., 2012). In Parodon moreirai and P. hilarii, the Z chromosome presents satellite DNA at the terminal region of the q arm, while the W chromosome has pPh2004 in the PRA (pseudo-autosomal region located at p arm), and WAp and GATAn sequences at the WSR (W specific region located at q arm). The W chromosome of Apareiodon sp. differs by the exclusive localization of the satellite pPh2004 in the q arm. However, the WSR of Apareiodon sp. keeps its repetitive sequences concerning the WAp probe identical to other Parodontidae, with exception of the GATAn expansion, which does not show accumulation in this chromosome.

In addition to the exclusive condition of the pPh2004 site in the q arm in the W chromosome of Apareiodon sp., this species possesses a high number of pPh2004 sites (14) distributed in three autosomal and ZW chromosomes. Karyotypes presenting numerous pPh2004 sites were described for P. nasus, P. hilarii, and A. affinis (Centofante et al., 2002; Vicente et al., 2003; do Nascimento et al., 2018), but the chromosomal sites localization is quite divergent in relation to Apareiodon sp. (Aripuaná River). All these cytogenetic features corroborate with the barcode, haplotypic network, and BAPs data, which demonstrate differentiation and absence of gene flow between Apareiodon sp. and the other Apareiodon species with a similar body color pattern.

DNA barcode studies have been useful in demonstrating genetic divergence among Parodonidae species, such
as *A. affinis*, *A. ibitiensis*, *A. piracicabae*, *A. vitattus*, *A. vladii*, *Apareiodon* sp. from Verde River, *P. nasus* and *P. moreirai* (Bellafronte et al., 2013), all well morphologically supported clades. The comparative population studies of *A. affinis* were equally informative, evidencing that the species from the upper Paraná differ from those of the lower Paraná River basin, and that three populations from the lower Paraná system (Uruguay, Paraguai, and Cuiabá rivers) display divergent chromosomal features, as well as low values of genetic divergence, which could indicate possible parapatric speciation processes in progress (do Nascimento et al., 2018). In our study, *Apareiodon* sp. represents the only Amazonian species (Madeira basin) that was compared to other *Apareiodon* species with a similar color pattern (*A. piracicabae*, *A. vitattus*, *A. affinis*), all of them from the Paraná River basin, and the results showed that the species belong to an undescribed species in the literature.

According to Lundberg (1998), the modern separation between the Paraná and Amazon systems occurred about 30 Myr ago. Since then, headwater captures among rivers from these hydrographic systems were also documented to have occurred during the last 10 Myr (Lundberg, 1998). The results of K2P genetic divergence demonstrated that *Apareiodon* sp. is distant by 20-23% from other *Apareiodon* evaluated, these high K2P values being compatible with distinct fish species (Hebert et al., 2003; Ward et al., 2005, 2009). Thus, although probable fauna interchanges occurred between the Paraná and Amazon systems, the population structure analysis, in addition to K2P genetic divergence evidenced that *Apareiodon* sp. appears as a distinct biological entity inside Parodontidae.

The haplotype network shows no overlap between *Apareiodon* sp., *A. vitattus*, *A. piracicabae*, and *A. affinis* COI haplotypes, corroborating absence of gene flow and isolation among these species. A similar case was demonstrated for *Leporinus friderici* as a new MOTU in the Madeira basin, which also demonstrated gene flow isolation to other *Leporinus friderici* in the Amazon and Paraná basins (Silva-Santos et al., 2018). Within the Amazon basin itself, *Apareiodon* sp. seems to be morphologically different from congener species and apparently it is not found outside the Madeira River basin (Pedroza et al., 2012; Fernandes et al., 2013).

Our phylogenetic analyses showed that specimens, although morphologically similar, compose particular groups in distinct branches with high support values, indicating clades that have ancient segregations. The close relationship of *Apareiodon* sp. and *A. affinis*, from the Paraná-Paraguai system, indicates some current or recent connections between the basins, corroborating data by Brea and Zuco (2011) and Dagosta and De Pinna (2017). In this scenario, population genetics data, in addition to particular cytogenetic characteristics, indicate that *Apareiodon* sp. from the Aripuanã River differentiated from morphologically close congeneric species and appears as a new Molecular Operational Taxonomic Unit (MOTU) within the Parodontidae.

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**Conflict of interest**

The authors have no conflicts of interest to declare.

**Author contributions**

EOS, CSP, OMF, MRV conceived and designed the study; EAO, HPS, OMF collected the samples; HPS, CSP analyzed the morphological data; EOS, GAD, EZO, VN, MRV conducted the cytogenetic experiments; GAD, RBA, MRV analyzed the COI data; EOS, GAD, RBA, HPS were involved in the manuscript draft; VN, CSP, MMC, LACB, OMF, MRV have contributed to the planning of the study and manuscript writing; all authors read and approved the final version.

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