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Morphological, histopathological and molecular assessments of *Prosorhynchoides* sp. (Digenea: Bucephalidae) in *Perna perna* (Bivalvia: Mytilidae) mussels sampled off the coast of Rio de Janeiro, southeastern Brazil

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ABSTRACT

Mussel production is expanding worldwide, and in Brazil the main species currently produced is the mussel *Perna perna*. Bucephalid trematodes have been recorded in *P. perna* but their larval identification is problematic. In this context, the aims of this paper were to evaluate the prevalence of bucephalids in *P. perna*, perform taxonomic and phylogenetic trematode studies, and analyze potential histopathological alterations in the infected host. Mussels obtained by fishers from Guanabara Bay, Rio de Janeiro, Brazil were weighed and measured, and internal organ tissues and parasites were collected. Of the 69 analyzed mussels, 24.6 % (17/69) were parasitized by bucephalid larvae. Sporocysts were located mainly in host mantle. Mussels presented sporocysts and cercaria within the connective tissue of mantle, all without associated inflammatory reactions. Parasite loads varied from less than 5 % to > 50 % of parasitized tissue. Histopathological examinations indicated that male or female gonads were not observed in 77 % (10/13) of parasitized mussels and in 4 % (2/56) identified as non-parasitized in the histology but previously classified as parasitized in the stereomicroscopic analysis. Thus, the absence of gonads may be associated with parasitism. *Prosorhynchoides* sp. is reported herein for the first time in mussels sampled on the coast of Rio de Janeiro, with genetic and histological data reported for the intermediate host, sporocysts and cercariae. New 28S rDNA, 18S rDNA and ITS1, 5.8S and ITS2 sequences are provided.

1. Introduction

Perna perna (Linnaeus, 1758) is a common species widely distributed along the southern and southeastern Brazilian coast, despite being a native African species (Marques, 1998; Resgalla Jr et al., 2008). This bivalve, also known as the brown mussel, is noteworthy for its high commercial sales and human consumption, mainly in traditional fishing communities (Resgalla Jr et al., 2008).

The gradual expansion of *P. perna* farming has exposed the need for studies concerning the occurrence of pathogens, as the knowledge of marine helminths, especially larval stages, is still limited (Carneiro-Schaefer et al., 2017). According to Selbach et al. (2022) research on aquatic ecosystems and, in particular, aquatic parasites are under-represented in the One Health strategies that link human, animal, and environmental health. Therefore, studies on larval helminths from marketed mussels are essential to add information on the biodiversity of marine parasites, as well as to prevent diseases in the cultivation areas.

Bivalves are known as intermediate trematode hosts, in which larval forms such as sporocysts and cercariae develop before reaching a definitive host, usually fish (Gibson, 2002; Marchiori et al., 2010). Bucephalid trematodes parasitize bivalves and their larvae are considered the most deleterious metazoan parasites to marine bivalves (Lauckner, 1983; Cribb et al., 2001). Among the Bucephalidae Poche, 1907, four genera were previously reported from marine fishes of South

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America: *Bucephalus* Baer, 1827, *Prosorhynchoides* (Dollfus, 1929), *Prosorhynchus* (Odhner, 1905) and *Rhipidocotyle* (Diesing, 1858) (Kohn et al., 2007; Muñoz and Bott, 2011; Pedro et al., 2016). In mussels, infections caused by larvae of *Bucephalus* sp. are considered common in *P. perna* from southern and southeastern Brazil (Umiji et al., 1976; Carneiro-Schaefer et al., 2017), and may compromise mussel development, leading to castration (Magalhães, 1998; Silva et al., 2002). However molecular analyses have not yet been performed in samples from Brazil.

Sporocysts and cercariae can be identified by stereomicroscopic analyses. However, the diagnosis may be uncertain when infection is still in its early stages. Therefore, histopathological studies are suggested as an additional method to classify the degree of host infection and avoid possible errors due to stereomicroscopic analyses only (Magalhães, 1998; Garcia and Magalhães, 2008). Previous records employing this complementary analysis reported 8.7 to 10.8 % increases in the number of parasitized bivalves compared to routine visual detection (Magalhães, 1998; Cochôa and Magalhães, 2008; Carneiro-Schaefer et al., 2017).

Bucephalid larvae cannot be identified at the genus or species level based on morphological characteristics, due to the lack of taxonomic characters that only appear in the adult form. Therefore, some authors prefer to identify trematodes found in bivalves only as bucephalids (Lasiak, 1993; Calvo-Ugarteburu and McQuaid, 1998a; Loureiro et al., 2001). In this regard, molecular techniques may represent a useful alternative for taxon identification (Eydal et al., 2013).

In this context, the aims of this paper were to evaluate the prevalence of larval trematodes in *P. perna* mussels from Guanabara Bay, Rio de Janeiro, Southeastern Brazil and perform taxonomic and phylogenetic assessments. Potential histopathological alterations in the mussels due to parasitism were also evaluated.

2. Material and methods

2.1. Sample collection and processing

Sixty-nine *P. perna* specimens were sampled from Guanabara Bay near Jurujuba Beach, in the municipality of Niterói (Rio de Janeiro, Brazil) (22°55.53′'S, 43°06.35′'W), from January to November 2019 and January 2020 (nine samplings). The mussels were collected by fishers from cultivation areas and adjacent rocky shores. The samples were numbered, packed in polystyrene boxes containing ice, and transported to the laboratory for processing. The fresh mussels were weighed with their shells, measured and divided into seven size classes, comprising 9 mm intervals from 60 to 129 mm in length. The valves were opened with the help of a blade and sex was determined through macroscopic mantle and gonad evaluations according to Lunetta (1969) and Resgalla Jr et al. (2008). Additionally, four *Pomatomus saltatrix* (Linnaeus, 1766), one *Micropogonias furnieri* (Desmarest, 1823) and one *Caranx latus* Agassiz, 1831 collected in the same area were examined in search of related adult bucephalid parasites.

2.2. Stereomicroscopic analyses

The internal organs of the mussels were examined under a stereomicroscope in a saline solution (0.7 %) for tissues and parasite sampling. The tissues were also squeezed between glass plates in saline medium for the detection of internal parasites. Detected sporocysts were ruptured with histological needles for cercariae release. Larvae were fixed in 70 % ethanol for morphological studies or frozen for molecular analyses. The 70 % ethanol-preserved specimens were stained using Semichon's carmine or Gomori's trichrome and mounted in Canada balsam. Measurements are presented in micrometers, with the range followed by the mean in parentheses. Mussel mantles were also frozen for molecular analyses. The sampled fish guts were also examined in saline medium under a stereomicroscope.

2.3. Genetic and phylogenetic analysis

Genomic mussel and sporocyst DNA were extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. The ITS1, 5.8S and ITS2 rDNA mussel regions were amplified using the BD1 (5′-GTCGTAACAAGGTTTCCGTA-3′) and BD2 (5′-TATGCTTAARTTCAGCGGGT-3′) (Luton et al., 1992) primers. The LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) primers were used for mitochondrial cytochrome oxidase c subunit 1 gene (COI) amplification (Folmer et al., 1994). For the sporocysts, the ITS1, 5.8S and ITS2 rDNA regions were also amplified using BD1 and BD2, while the partial 18S rDNA was amplified using the SB3a (5′-GGAGGG-CAAGTCTGGTGC-3′) and A27a (5′-CCATACAAATGCCCCCGTCTG-3′) (Hall et al., 1999) primers and the 28S rDNA was amplified using the LSU5 (5′-TAGGTCGACCCGCTGAAYTTAAGCA-3′) and 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′) primers (Tkach et al., 2003). PCRs were carried out using cycling parameters as previously described by these authors. The PCR products were analyzed by electrophoresis employing 1.5 % agarose in Tris-borate Ethylenediamine tetraacetic acid (EDTA) gels stained with SybrGreen DNA gel Stain (Invitrogen, Eugene, Oregon, USA) and photographed under ultraviolet trans-illumination. PCR amplicons were purified using the ExoSap-IT (USB® Products Affymetrix Inc., Cleveland, Ohio, USA). DNA cycle sequencing reactions were performed using the BigDye Terminator v.3.1 (Applied Biosystems, Foster City, CA, USA) and automated sequencing was carried out using the Sequencing Platform at the Fundação Oswaldo Cruz (PDTIS/Fiocruz), in Brazil. Newly generated sequences of both strands were verified and edited using the MEGA software version X (Kumar et al., 2018). Sequences were compared to others available in the GenBank database employing the BLAST program from the National Center for Biotechnology Information (NCBI) server (https://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1990). Evolutionary divergence estimates between sequences were conducted in MEGA X using the Kimura 2-parameter (K2p) model (Tamura et al., 2004).

In order to explore the phylogenetic relationships of each DNA region, nucleotide sequences were aligned with the CLUSTALW algorithm using the MEGA X (Thompson et al., 1994). Bayesian inference (BI) phylogenetic trees were conducted using Monte Carlo Markov Chain (MCMC) analysis available in the BEAST v2.6.3 software (Bouckaert et al., 2019). The likelihood parameters set for the BI analysis were based on the Akaike Information Criteria (AIC) test in jModelTest2 (Nylander, 2004). The selected model was the General Time-Reversible (GTR) for 28S, and the Hasegawa-Kishino-Yano (HKY) for the 18S and ITS1, 5.8S and ITS2 regions, both employing the birth–death model (BDM). Posterior probabilities (pp) were calculated via 10,000,000 generations, sampling every 1,000th tree being saved. Tracer v1.7.2 (Rambaut et al., 2018) was used to validate the convergence and mixing to ensure all effective sample size (ESS) values>200. Trees were presented as Maximum-Clade Credibility (MCC) trees using the TreeAnnotator v2.6.3 software after discarding the first 10 % as burn-in, and visualized using the FigTree v1.4.4 program (Rambaut, 2018). For tree rooting, the best sequences used as outgroups were *Olssonium turneri* Bray & Gibson, 1980 and *Pleorchis uku* Yamaguti, 1970 for 28S rDNA, *Proctoeces lintoni* Siddiqi & Cable, 1960 and *Proctoeces maculatus* (Looss, 1901) for 18S rDNA and *Pseudolepidapedon balistis* Manter, 1940 for the ITS1, 5.8S and ITS2 regions. The GenBank sequences used for the phylogenetic analysed are listed in Table 1.

2.4. Histological processing

The mid-parts of mussel mantles were fixed in alcohol 70 % or Carson's Millonig formalin for routine histological procedures. The tissue blocks were embedded in paraffin and serial sections (5 µm) were cut and mounted on non-silanized slides which were then stained with hematoxylin-eosin (HE) (Carson and Cappellano, 2015). The presence of

Table 1
Digeneans used in the phylogenetic analyses with their respective GenBank accession numbers.

Species	28S rDNA	18S rDNA	ITS1-5.8S-ITS2 rDNA
Bucephalus cynoscion Hopkins, 1956	KT273397	–	KT273396; KT273397
Bucephalus gorgon (Linton, 1905)	KT273400	–	KT273400
Bucephalus margaritae Ozaki & Ishibashi, 1934	KT273395	–	KT273395
Bucephalus polymorphus von Baer, 1827	AY289248	–	AY289241
Dicrogaster contracta Looss, 1902	–	FJ211255; FJ211256	–
Dollfustrema durum Nolan, Curran, Miller, Cutmore, Cantacessi & Cribb, 2015	KT213572	–	–
Dollfustrema hefeiense Liu in Zhang et al., 1999	KT273386	–	KT273386
Dollfustrema vaneyi (Tseng, 1930)	–	–	EF198191
Grammatorcynicola brayi Bott & Cribb, 2005	KT213573	–	–
Grammatorcynicola nolani Bott & Cribb, 2005	KT213574	–	–
Heterobucephalopsis perardua Nolan, Curran, Miller, Cutmore, Cantacessi & Cribb, 2015	KT213571	–	–
Heterobucephalopsis yongi Cutmore, Nolan & Cribb, 2018	MH754949	–	–
Megasolena sp.	–	JQ782538	–
Olssonium turneri Bray & Gibson, 1980	AY222283	–	–
Parabucephalopsis parasiluri Wang, 1985	AB640884	–	–
Paurorhynchus hiodontis Dickerman, 1954	KT273401	–	–
Pleorchis uku Yamaguti, 1970	DQ248216	–	–
Proctoeces lintoni Siddiqi & Cable, 1960	–	JQ782521	–
Proctoeces maculatus (Looss, 1901)	–	AY222161	–
Prosorhynchoides sp.	LC498576	JQ782530; JQ782531; JQ782533	–
Prosorhynchoides apogonis Bott & Cribb, 2005	KT213576	–	–
Prosorhynchoides borealis Bartoli, Gibson & Bray, 2006	–	JN182208	JN182210; JN182212
Prosorhynchoides caecorum (Hopkins, 1956)	KT273393	–	KT273392
Prosorhynchoides carvajali Muñoz & Bott, 2011	–	JQ782535	–
Prosorhynchoides cutmorei Hammond, Cribb & Bott, 2018	MG953232	–	–
Prosorhynchoides galaktionovi Hammond, Cribb, Nolan & Bott, 2019	MN310396	–	–
Prosorhynchoides gracilescens (Rudolphi, 1819)	AY222224	AJ228789	–
Prosorhynchoides longoviferus (Manter, 1940)	KT273387	–	KT273387
Prosorhynchoides megacirrus (Riggin & Sparks, 1962)	KT273391	–	KT273391
Prosorhynchoides kohnae Hammond, Cribb, Nolan & Bott, 2019	MN310397	–	–
Prosorhynchoides moretonensis Hammond, Cribb & Bott, 2018	MG953230	–	–
Prosorhynchoides ovatus (Linton, 1900)	KT273399	–	KT273399
Prosorhynchoides ozakii (Nagaty, 1937)	AB640885	–	–
Prosorhynchoides paralichthydis (Corkum, 1961)	KT273398	–	KT273398
Prosorhynchoides scomberomorus (Corkum, 1968)	KT273389	–	KT273388
Prosorhynchoides waeschenbachae Hammond, Cribb & Bott, 2018	MG953231	–	–
Prosorhynchus longisaccatus Durio & Manter, 1968	KT213575	–	–
Prosorhynchus pacificus Manter, 1940	KT273385	–	KT273385
Pseudolepidapedon balistis Manter, 1940	–	–	KJ820760
Rhipidocotyle angusticollis Chandler, 1941	KT273383	–	KT273383
Rhipidocotyle campanula (Dujardin, 1845)	JQ346713	–	–
Rhipidocotyle fennica Gibson, Taskinen & Valtonen, 1992	KM068119	–	–
Rhipidocotyle galeata (Rudolphi, 1819)	AY222225	AY222119	–
Rhipidocotyle lepisostei Hopkins, 1954	KT273390	–	–
Rhipidocotyle transversale Chandler, 1935	KT273394	–	KT273394
Rhipidocotyle tridecapapillata Curran & Overstreet, 2009	KT273384	–	–
Saccocoelium brayi Blasco-Costa, Balbuena, Raga, Kostadinova & Olson, 2010	–	FJ211227	–
Saccocoelium tensum Looss, 1902	–	FJ211252	–

sporocysts and cercariae in tissues were evaluated under a Leica DM LS2 microscope. Parasite load was analyzed semi-quantitatively using a light microscope. All the microscopic fields were examined using an objective of 10x and parasite load was expressed as the percentage of the entire histological section of the mantle that was parasitized using an adapted classification of Garcia and Magalhães (2008), without quantification by the Weibel graticule. Parasite load was classified as mild (less than 5 % of the entire histological section of the mantle was parasitized), moderate (higher than 5 % and less than 50 % of the entire histological section of the mantle was parasitized) or high (>50 % of the entire histological section of the mantle was parasitized).

3. Results

The 69 analyzed mussels ranged from 60 to 121 (86.1 ± 14.4) mm in total length and weighed 15.5 to 113.6 (49.3 ± 21.9) g. A total of 36 females and 32 males were analyzed. One individual could not be sexed. The rDNA analysis confirmed the host species to be *P. perna* and new ITS1, 5.8S and ITS2 regions and COI sequences were deposited in GenBank under accession numbers OK560853 and OK559744, respectively.

The mussel stereomicroscope analysis indicated that 16 out of 69 mussels (23.2 %) were infected with orange/red filamentous sporocysts. Some sporocysts were less voluminous and displayed a less intense pigmentation than others. Thirteen of the infected mussels were males, two were females and one was undetermined (Fig. 1A). The infection sites comprised the mantle 100 % (16/16), visceral mass 43.7 % (7/16), gills 37.5 % (6/16), digestive gland 37.5 % (6/16) and muscle tissue 31.2 % (5/16). In comparison, histopathological analysis indicated 13 parasitized individuals (18.8 %), 12 determined as previously infected in the stereomicroscopic analysis (nine males, two females, and one undetermined). Parasite mantle loads were variable in the 13 parasitized mussels detected in the histopathological examination, nine exhibiting over 50 % (high) of parasitized tissue and three with 5 to 50 %

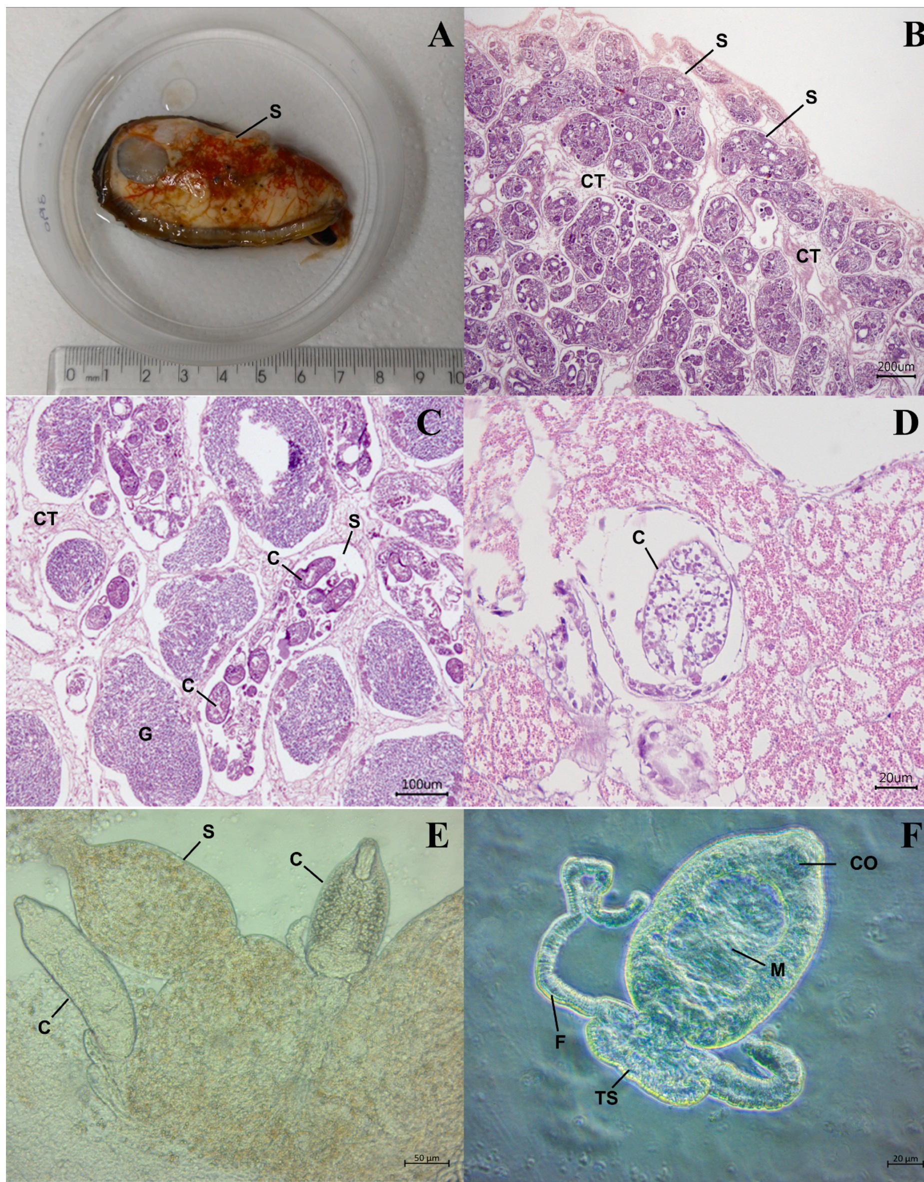

Fig. 1. Micrographs of larval *Prosorhynchoides* sp.: A- Filamentous sporocysts in the mantle of a male *Perna perna*. B–D- Histological findings in the mantle of mussels parasitized by *Prosorhynchoides* by hematoxylin-eosin staining. B- Male with over 50% of the connective mantle tissue parasitized by trematode sporocysts. Absence of inflammatory reactions associated with parasitism and gonads. C- Male with over 50% of the connective mantle tissue parasitized by trematode sporocysts. Detail of sporocysts containing multiple cercariae in the connective tissue between the gonads. No inflammatory reaction associated with the detected parasitism is observed. D- Cercaria in the connective mantle tissue of a female mussel with less than 5% of parasitized mantle. No inflammatory reaction associated with the detected parasitism is observed. E- Cercaria emerging from sporocysts. F- Fully developed infective cercaria. Abbreviations: connective tissue (CT), sporocysts (S), gonads (G), cercariae (C), cephalic organ (CO), mouth (M), tail stem (TS) and furca (F).

(moderate) of parasitized tissue. Additionally, one female not previously detected as infected in the stereomicroscopic analysis exhibited a mild parasite load (less than5%) as revealed by histopathology (Fig. 1B – D). The stereomicroscopic analysis detected four positive cases not detected by histopathology. Increasing parasite loads led to increased mollusk tissue replacement by sporocyst masses. Therefore, considering both analysis methods (histopathological and stereomicroscopic), the overall infection prevalence was of 24.6 % (17/69), 13 males (76.5 %), 3 females (17.6 %), and 1 undetermined (5.9 %). The total shell length of all parasitized mussels ranged from 77 to 121 (91.8 ± 11.5) mm, with the

Table 2
Parasitized *Perna perna* mussels according to shell size class.

Size class (mm)	Number of parasitized mussels
60 – 69	0
70 – 79	2
80 – 89	6
90 – 99	5
100 – 109	3
110 – 119	0
120 – 129	1

highest prevalences of parasitized mussels in the 80 to 89 and 90 to 99 size classes (Table 2). The histopathological examination indicated trematode sporocysts and/or cercariae within the connective tissue of mussel mantles without associated inflammatory reactions. Male or female gonads were not observed in 77 % (10/13) of parasitized mussels, eight (80 %) with over 50 % of parasitized tissue and two (10 %) with 5 to 50 % of parasitized tissue. Concerning the non-parasitized mussels, gonads were not observed in 4 % (2/56). These two individuals, however, were classified as parasitized in the stereomicroscopic analysis.

Mussel sporocysts were filamentous, with irregular swellings with long tailed transparent cercariae at different developmental stages (Fig. 1B – E); cercariae, dorsoventrally flattened, measured 147 × 94 µm; cephalic organ elongated; tail stem at the posterior end of body measuring 36 × 80 µm; forked tail with lateral arms (furca) measured 240 µm when contracted, but sometimes extending far beyond the body length. The mouth at mid-body, surrounded by a small muscular pharynx, was compatible with Bucephalidae gen. sp. (Fig. 1F). The examined fish contained no bucephalid parasites.

In total, 11 mussel sporocyst sequences were obtained, two partial 28S rDNA (OK429320 and OK429321), six partial 18S (accession numbers OK429323, OK429324, OK429325, OK429326, OK429327

and OK429328) and three ITS1, 5.8S and ITS2 (OK429329, OK429330 and OK429331) sequences. Genetic divergence values estimated using the p-distance of the ITS1, 5.8S and ITS2 regions among our sequences were 0.2 % with OK429330 differing by two nucleotides. No intraspecific variation was noted for the 28S and 18S sequences (Table 3). The rDNA analyses of both sporocysts and cercariae obtained in this study were compatible with species belonging to the *Prosorhynchoides* genus.

The closest 28S rDNA sequence in GenBank according to BLAST was *Prosorhynchoides moretonensis* Hammond, Cribb & Bott, 2018, with a 97.2 % identity. The K2p distance was 2.9 % with 30 divergent nucleotides in a 1059 bp. Sequence for *Prosorhynchoides paralichthydis* (Corkum, 1961), although with 96.5 % similarity, displayed a lower K2p distance (2.4 %). For the partial 18S rDNA sequence, the identity with *Prosorhynchoides borealis* Bartoli, Gibson & Bray, 2006 was of 98.8 % for 415 bp with a K2p distance of 1.2 % (5 divergent nucleotides). For the ITS1, 5.8S and ITS2 rDNA, *P. paralichthydis* displayed a K2p distance of 6.0–6.1 %, with 69 divergent nucleotides and 93.7–93.8 % identity for 972 bp. *Prosorhynchoides borealis* exhibited a K2p distance ranging from 5.6 to 8.0 % (Table 3).

The Bayesian phylogenetic 18S rDNA tree indicated that *P. borealis* and *Prosorhynchoides gracilescens* (Rudolphi, 1819) were the closest species to the new sequence, with a node support of one (Fig. 2). BI analyses of the 28S rDNA and ITS1, 5.8S and ITS2 yielded similar topologies, with the new sequences forming a clade with *Prosorhynchoides ovatus* (Linton, 1900), *P. paralichthydis* and *Bucephalus gorgon* (Linton, 1905). However, in the ITS1, 5.8S and ITS2 phylogenetic tree, the new sequences and *P. paralichthydis* were even more closely related, with a node support of 0.97 (Figs. 3–4). Species belonging to the *Prosorhynchoides*, *Bucephalus* and *Rhipidocotyle* genera were grouped in different clades and almost all branches were well-supported, representing polyphyletic groups. No identical sequence for the sporocyst studied herein was found in the GenBank database and the BI analysis indicated this as a novel species occurring off the coast of Rio de Janeiro.

4. Discussion

Perna perna is the largest Brazilian mytilid, potentially reaching 200 mm in shell length (Ferreira and Magalhães, 2004; Resgalla Jr et al., 2008). All sampled *P. perna* individuals were over 50 mm in length and, according to the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA, 2006), classified as adults. Larger, and therefore, older mussels, are more likely to harbour bucephalid infections (Lasiak, 1993). This is corroborated by our data, where the highest parasitized size classes were 80 to 89 mm and 90 to 99 mm. However, differences related to size classes have been reported by Magalhães (1998), who identified parasitized mussels from the 30 to 40 mm size class, gradually increasing the number of parasitized individuals up to the 60 to 70 mm class, and declining in larger size class.

The intense orange to red coloration in the internal organs of male mussels makes it easy to macroscopically distinguish between infected

Table 3
Genetic divergence (Kimura-2-parameter, expressed as percentages) estimated using the 28S, 18S and ITS1, 5.8S and ITS2 rDNA regions among *Prosorhynchoides* species retrieved from GenBank and the new sequences reported in the present study, represented by (*).

28S rDNA																
OK429320 *Prosorhynchoides* sp. (*)																
OK429321 *Prosorhynchoides* sp. (*)	0.0															
KT273398 *Prosorhynchoides paralichthydis*	2.4	2.4														
MG953230 *Prosorhynchoides moretonensis*	2.9	2.9	3.6													
MG953231 *Prosorhynchoides waeschenbachae*	3.0	3.0	3.3	0.9												
MN310397 *Prosorhynchoides kohnae*	3.3	3.3	3.5	1.4	1.1											
MG953232 *Prosorhynchoides cutmorei*	3.3	3.3	3.5	1.8	1.5	1.0										
AY222224 *Prosorhynchoides gracilescens*	3.5	3.5	3.6	3.9	3.4	4.2	4.0									
MN310396 *Prosorhynchoides galaktionovi*	3.5	3.5	3.5	1.6	1.3	0.7	1.2	4.0								
KT273399 *Prosorhynchoides ovatus*	4.1	4.1	4.6	5.1	5.1	5.7	5.6	4.9	5.3							
LC498576 *Prosorhynchoides* sp.	5.5	5.5	5.9	5.3	5.0	5.4	5.4	5.4	5.4	7.2						
KT273391 *Prosorhynchoides megacirrus*	7.3	7.3	7.1	7.1	7.0	7.4	7.4	7.2	7.3	8.2	5.1					
KT273393 *Prosorhynchoides caecorum*	8.0	8.0	7.7	7.2	7.2	7.6	7.6	7.5	7.5	8.1	5.6	1.5				
AB640885 *Prosorhynchoides ozakii*	8.6	8.6	7.9	7.7	7.6	8.0	8.0	8.6	7.9	9.8	6.9	6.2	7.0			
KT273389 *Prosorhynchoides scomberomorus*	9.6	9.6	8.8	8.7	8.9	9.4	9.5	9.4	9.5	10.5	8.0	6.7	6.5	6.1		
KT273387 *Prosorhynchoides longoviferus*	9.9	9.9	9.4	9.4	9.3	10.3	10.3	10.5	10.1	10.9	8.4	7.4	8.3	6.9	6.1	
KT213576 *Prosorhynchoides apogonis*	10.0	10.0	9.7	10.4	10.4	10.6	10.3	10.8	10.6	11.2	10.4	12.7	12.7	13.0	12.7	13.4
18S rDNA																
OK429323 *Prosorhynchoides* sp. (*)																
OK429324 *Prosorhynchoides* sp. (*)	0.0															
OK429325 *Prosorhynchoides* sp. (*)	0.0	0.0														
OK429326 *Prosorhynchoides* sp. (*)	0.0	0.0	0.0													
OK429327 *Prosorhynchoides* sp. (*)	0.0	0.0	0.0	0.0												
OK429328 *Prosorhynchoides* sp. (*)	0.0	0.0	0.0	0.0	0.0											
JN182208 *Prosorhynchoides borealis*	1.2	1.2	1.2	1.2	1.2	1.2										
AJ228789 *Prosorhynchoides gracilescens*	1.5	1.5	1.5	1.5	1.5	1.5	0.2									
JQ782535 *Prosorhynchoides carvajali*	5.3	5.3	5.3	5.3	5.3	5.3	5.8	6.1								
JQ782533 *Prosorhynchoides* sp.	6.3	6.3	6.3	6.3	6.3	6.3	6.9	7.1	1.5							
JQ782531 *Prosorhynchoides* sp.	6.6	6.6	6.6	6.6	6.6	6.6	7.1	7.4	1.7	0.2						
JQ782530 *Prosorhynchoides* sp.	6.9	6.9	6.9	6.9	6.9	6.9	7.4	7.7	2.0	0.5	0.2					
ITS1-5.8S-ITS2 rDNA																
OK429329 *Prosorhynchoides* sp. (*)																
OK429330 *Prosorhynchoides* sp. (*)	0.0															
OK429331 *Prosorhynchoides* sp. (*)	0.2	0.2														
JN182212 *Prosorhynchoides borealis*	5.6	5.6	5.6													
KT273398 *Prosorhynchoides paralichthydis*	6.0	6.0	6.1	6.5												
JN182210 *Prosorhynchoides borealis*	8.0	8.0	7.9	0.0	7.7											
KT273399 *Prosorhynchoides ovatus*	9.3	9.3	9.4	7.1	9.0	8.4										
KT273391 *Prosorhynchoides megacirrus*	14.8	14.8	14.8	12.1	12.6	13.9	16.0									
KT273393 *Prosorhynchoides caecorum*	17.2	17.2	17.1	13.8	15.2	15.9	17.2	4.6								
KT273387 *Prosorhynchoides longoviferus*	17.4	17.4	17.5	13.1	16.3	15.9	17.8	13.7	16.0							
KT273388 *Prosorhynchoides scomberomorus*	18.7	18.7	19.0	13.1	17.0	16.5	17.8	15.9	17.1	11.4						

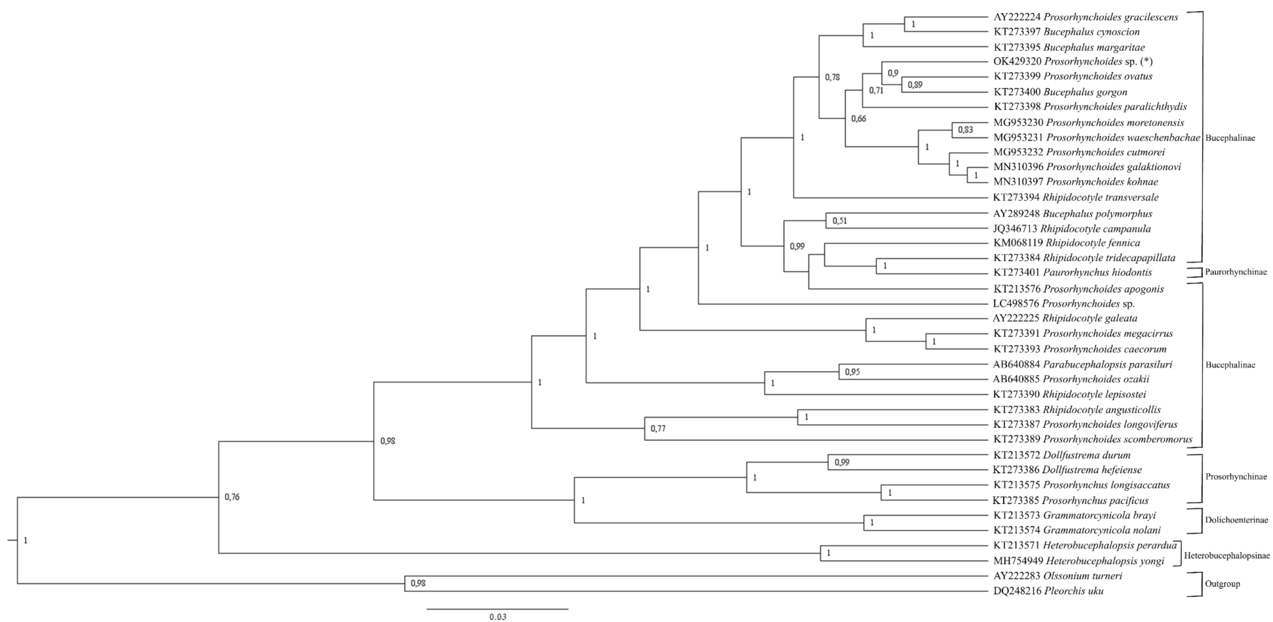

Fig. 2. Bayesian phylogenetic topology of Bucephalinae and closely related subfamilies constructed with the 28S rDNA gene dataset. Support values at branching points are shown as Bayesian posterior probabilities (>0.5). The new sequence is highlighted with (*).

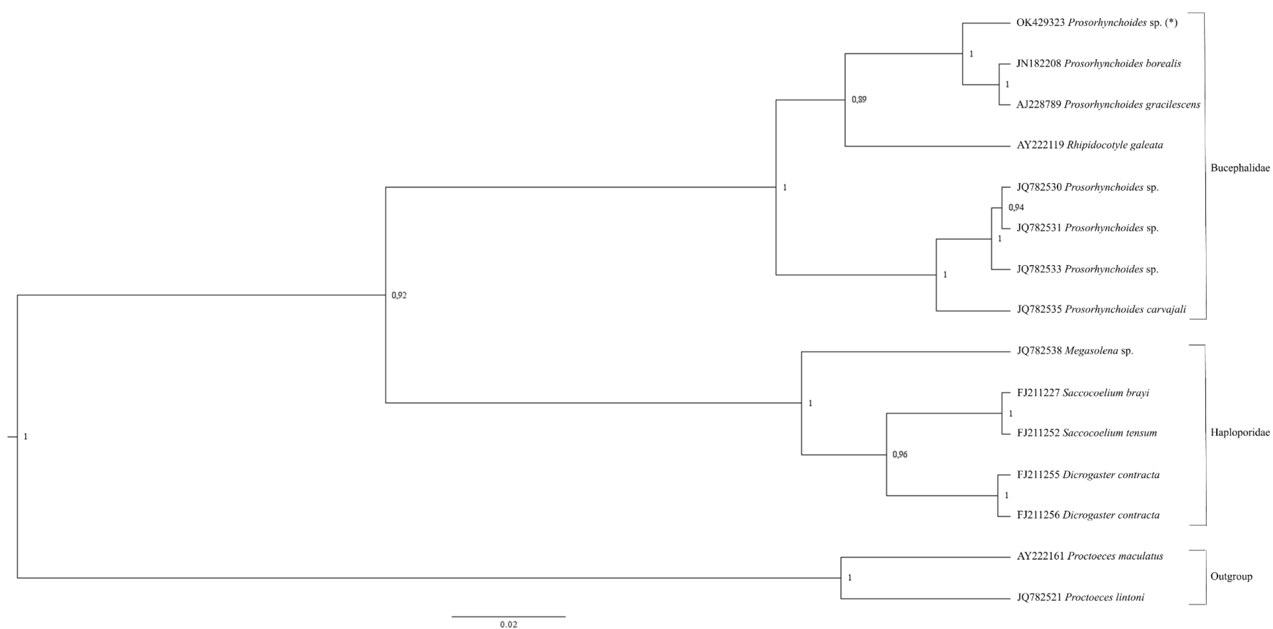

Fig. 3. Bayesian phylogenetic topology of Bucephalidae and closely related family construted with the 18S rDNA gene dataset. Support values at branching points are shown as Bayesian posterior probabilities (>0.5). The new sequence is highlighted with (*).

and uninfected individuals. However, a more careful evaluation is necessary in the case of females as, during their reproductive period, male *P. perna* mussels display a creamy white mantle color, and females, orange (Lunetta, 1969; Resgalla Jr et al., 2008). The mussel of undetermined sex exhibited a transparent mantle, probably associated to the follicle emptying stage, as reported by Lunetta (1969), or due to parasitic castration (Magalhães, 1998), as this individual was highly parasitized.

Lauckner (1983) reported that both male and female bivalve hosts are usually infected and castrated by bucephalids at similar rates and that sporocysts can infiltrate practically every organ except for the foot, forming a densely interwoven network. All parasitized *P. perna* exhibited mantle sporocysts and cercariae with optional infection foci in other organs, except for the foot. Lasiak (1993) also reported the mantle as the main source of infection, affecting other organs in more advanced infection stages. Although the number of female *P. perna* (36) collected in this study was slightly higher than males (32), males exhibited the highest infection rate (13 × 3 infected specimens), in accordance to Addum and Oliveira (2010) who also observed higher bucephalid prevalence in males. According to Magalhães (1998), parasitic preference for the mantle and the digestive gland of mussels during the early stages of the parasite biological cycle indicate the significant trematode need for nutritious substances.

The parasitological stereomicroscope observations indicated 16 parasitized mussels, while only 12 were confirmed in the histopathological analysis, probably due to restricted paraffin sample sizes.

Fig. 4. Bayesian phylogenetic topology of Bucephalinae and closely related subfamily construted with the ITS1, 5.8S and ITS2 regions dataset. Support values at branching points are show as Bayesian posterior probabilities (>0.5). The new sequences are highlighted with (*).

However, one positive histopathology assessment was negative at first view in the stereomicroscopic analysis, due to mild parasitic loads. Although the stereomicroscope observations indicated higher number of parasitized specimens, the histopathology is important to confirm mild infections not seen in previous analyses. These complementary methods are, therefore, important to better confirm sporocyst and cercariae parasitism in mussels.

Mytilid gonads are diffuse, occurring in the visceral mass and expanding into the mantle (Lunetta, 1969; Resgalla Jr et al., 2008). Although no associated inflammatory reaction was observed in the mantle of parasitized mussels, the intense parasitism observed in 69 % of the analyzed individuals may have contributed to absence of gonads in 80 % of these mollusks, in contrast with presence of gonads in 96 % of mussels not parasitized as revealed by the histopathological examination. This hypothesis is supported by other authors who have described the castration of parasitized mussels by *Bucephalus* spp. (Magalhães, 1998; Silva et al., 2002). Although the consumption of parasitized mussels by bucephalids is not harmful to humans (Resgalla Jr et al., 2008), possible evidence of *P. perna* castration may compromise population renewal in the investigated study area. In addition, the presence of bucephalids may also favor weakening of the adductor muscle in *P. perna*, which may compromise valves closure and make these bivalves more susceptible to some predators (Calvo-Ugarteburu and McQuaid, 1998b).

The trematode infection prevalence in *P. perna* observed herein was of 24.6 %, close to the value reported by Marchiori et al. (2010) (21.6 %), in the state of Santa Catarina, Brazil. Umiji et al. (1976) reported a higher prevalence ranging from 30 to 35 % in the nearby coast of the state of São Paulo. Nevertheless, this prevalence greatly differs from most reports for the Brazilian coast, which are generally lower, ranging from 1.6 to 16.1 % (Lima et al., 2001; Silva et al., 2002; Galvão et al., 2006; Cochôa and Magalhães, 2008; Garcia and Magalhães, 2008; Addum and Oliveira, 2010; Silva et al., 2012; Carneiro-Schaefer et al., 2017; Stakowian et al., 2020). Low prevalences varying between 1 and 12 % have also been reported for the coast of South Africa (Lasiak, 1993; Calvo-Ugarteburu and McQuaid, 1998a). Loureiro et al. (2001) noted an association between increased parasitic incidence and environmental pollutants in Guanabara Bay, in the state of Rio de Janeiro. Considering that all examined mussels were adults and that the study area displays signs of pollution (Fries et al., 2019), not only size but also water quality may be an interfering factor concerning the high prevalence of parasitism observed herein. Other factors can also impact susceptibility to infection, for example, the geographic variations. Mahony et al. (2021) indicated that trematode prevalence in cockles from five European sites was related to sea water temperature. Umiji et al. (1976) showed that *P. perna* from rocky shores exposed to wave impacts from the open ocean were non-parasitized. This may also explain the prevalence found here since Jurujuba Beach is more sheltered and calmer.

The species of Bucephalid reported parasitizing fish in South America include *Bucephalus australis* (Szidat, 1961) (Argentina), *Bucephalus margaritae* Ozaki & Ishibashi, 1934 (Brazil and Venezuela), *B. gorgon* (Chile and Colombia), *Bucephalus solitarius* Kohn, 1966 (Brazil); *Bucephalus urophyci* Szidat, 1961 (Argentina). Species of *Rhipidocotyle* reported include *Rhipidocotyle adbaculum* Manter, 1940 (Peru), *Rhipidocotyle santanaensis* Lunaschi, 2004 (Argentina and Brazil) and only from Brazil, *Rhipidocotyle angusticolle* Chandler, 1941, *Rhipidocotyle fluminensis* Vicente & Santos, 1973, *Rhipidocotyle gibsoni* Kohn & Fernandes, 1994, *Rhipidocotyle jeffersoni* (Kohn, 1970), *Rhipidocotyle pentagonum* (Ozaki, 1924), *Rhipidocotyle quadriculatum* Kohn, 1961. Species of *Prosorhynchoides* include *Prosorhynchoides arcuatus* (Linton, 1900) (=*Bucephalopsis callicotyle*) (Brazil), *Prosorhynchoides cambapuntaensis* Lunaschi, 2004 (Argentina), *Prosorhynchoides carvajali* Muñoz & Bott, 2011 (Chile), *Prosorhynchoides hemirhamphi* (Fischthal & Nasir, 1974) (Venezuela) and *Prosorhynchoides rioplatensis* (Szidat, 1970) (Argentina and Peru) (Kohn et al., 2007; Muñoz and Bott, 2011; Pedro et al., 2016). However, just some of these species have sequences available in GenBank for comparison.

The molecular data based on the 28S, 18S and ITS1, 5.8S and ITS2 regions indicate that the bucephalid larvae observed in *P. perna* is a *Prosorhynchoides* species. Currently, this genus comprises 80 accepted species (WoRMS, 2021), with *P. arcuatus* occurring in Rio de Janeiro (Kohn, 1962), reported in *P. saltatrix*. However, no sequences are available for the adult of this species for comparison to date. *Prosorhynchoides carvajali*, described in marine fishes from Chile, was probably incorrectly reported in freshwater fishes in the state of Amazonas (Brazil) (Fernandes et al., 2017). The phylogenetic 18S rDNA analysis included *P. carvajali* from Chile (Muñoz et al., 2015) and the few bucephalid sequences available in GenBank. The BI analysis considered *P. borealis* and *P. gracilescens* as the closest species compared to the analyzed *P. perna* parasite larva, while *P. carvajali* was set in a well-

differentiated clade. Previous reports of trematodes parasitizing *P. perna* in Brazil have been mainly related to *Bucephalus* sp. (Umiji et al., 1976; Lima et al., 2001; Silva et al., 2002; Galvão et al., 2006; Cochôa and Magalhães, 2008; Garcia and Magalhães, 2008; Addum and Oliveira, 2010; Silva et al., 2012; Carneiro-Schaefer et al., 2017; Stakowian et al., 2020). Marchiori et al. (2010) investigated the life cycle of a parasite and its intermediate and definitive hosts, identified as *B. margaritae*, through experimental studies and samplings conducted off the coast of Southern Brazil. To the best of our knowledge, no genetic sequences available for the specimens cited in those studies are available. A *B. margaritae* sequence from Mexico was included in the phylogenetic trees of the 28S and ITS1, 5.8S and ITS2 regions, although not close to our sequences. *Bucephalus varicus* Manter, 1940 was not included in the 28S rDNA tree, as it is considered a synonym of *B. margaritae* after WoRMS (2021). ITS sequences are used to explore the validity of species and genetic variations of less than 1.0 % between digenean species sequences are often considered intraspecific variations (Nolan and Cribb, 2005). Therefore, the three sequences obtained herein from the ITS1, 5.8S and ITS2 regions are considered the same species, as the observed genetic variation was of only 0.2 %.

The phylogenetic analyses of the molecular sequences obtained herein revealed that the trematode detected in *P. perna* is included in a well-supported clade with representatives of the Bucephalinae subfamily, including species belonging to the *Prosorhynchoides*, *Bucephalus* and *Rhipidocotyle* genera. A similar polyphyletic result in this subfamily was observed in the phylogenetic trees of 28S and ITS rDNA reported in other studies (Nolan et al., 2015; Hammond et al., 2020; Shirakashi et al., 2020). Nolan et al. (2015) concluded that there are clear issues with the circumscription of these genera, mainly due to the fact that some species were transferred from one of these three genera to another. This explains why the genera did not form monophyletic clades in phylogenetic trees.

The lack of knowledge of other hosts involved in the life cycle of this parasite in the investigated area and the absence of sequences of related adult form for comparison it impossible to identify to species level the *Prosorhynchoides* sp. larvae.

Nevertheless, this study contributes to the knowledge of new molecular sequences of *Prosorhynchoides* sp. that parasitizes *P. perna* in Guanabara Bay, Rio de Janeiro, located in the Southeastern coast of Brazil. Further studies concerning this trematode in potential hosts at this same location should be carried out in order to understand their life cycle and involved hosts.

5. Conclusions

Prosorhynchoides sp. is reported herein for the first time in mussels marketed off the coast of Rio de Janeiro alongside genetic and histological data for the intermediate host, sporocysts and cercariae. New gene sequences have been deposited in GenBank and will serve as comparisons for future research on adult parasites.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical standards

This study was authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, license no. 68263-1 and 68263-2) in accordance with the guidelines of the Brazilian College or Animal Experiments (COBEA).

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