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Prevalence and molecular characterisation of *Calyptospora* parasites Overstreet, Hawkins and Fournié, 1984 (Apicomplexa: Calyptosporidae) in fishes from the eastern Amazon, Brazil

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ABSTRACT

This work reports the occurrence of coccidia of the genus *Calyptospora* in fishes from the eastern Amazon. Fish were collected on flood plains in the municipality of Macapá, State of Amapá, Brazil. Fresh squash preparations of liver, heart and gallbladder were examined under light microscope. Positive samples of *Geophagus proximus* and *Hoplias malabaricus* were used to detect parasites by PCR with *Calyptospora*-specific primers mRF and mrR, which amplify a region of the 18S rRNA gene. Oocysts were observed in 55% of 130 fishes examined. Parasite prevalence varied according to feeding habits, and was 100% in carnivores, 74% in omnivores (invertivores and detritivores) and 0% in herbivores. Variation in the frequency of parasitized organs showed 100% in the liver, 30% in the gallbladder, and 9% in the heart. The sequences obtained from *G. proximus* and *H. malabaricus* were identical and showed 99% similarity to *Calyptospora serrasalmi*. To our knowledge, this is the first report of the occurrence of *Calyptospora* in 10 new species of fish from the region of the eastern Brazilian Amazon. The results demonstrate the occurrence of *C. serrasalmi* in the region and the research provides new primers for the diagnosis of *Calyptospora* spp.

1. Introduction

Coccidians are intracellular parasites belonging to the phylum Apicomplexa, which are described in all vertebrate classes [1]. Among the coccidia *stricto sensu* of marine and freshwater fish, four genera are known, *Calyptospora* Overstreet, Hawkins and Fournie, 1984, *Eimeria* Schneider, 1875, *Goussia* Labbé, 1896, *Crystallospora* Labbé, 1896, and *Cryptosporidium* Tyzzer, 1907 [2]. Their disease impact is poorly understood, but there is increasing evidence that they are potential pathogens [3].

The protozoan parasites of the genus *Calyptospora* have heteroxenous life cycles involving fishes and shrimp. Their oocysts are primarily confined to liver tissue, but may occur in other organs, such as the pancreas and gallbladder [1]. Little is known concerning the diversity of these parasites, and to date only five species have been formally described: *Calyptospora funduli* Duszynski et al. 1979; and *Calyptospora empristica* Fornie et al. 1985, recorded in the United States; and *Calyptospora serrasalmi* Cheung et al. 1986 in *Serrasalmus niger* Jardine 1841; *Calyptospora tucunarensis* Békési and Molnár 1991 in *Cichla ocellaris* Bloch and Schneider 1801; and *Calyptospora spinosa* Azevedo et al. 1993 described in *Crenicichla lepidota* Heckel 1840, recorded in Brazil. There are also records of the parasitism of *Calyptospora* sp. in *Arapaima gigas* Schinz, 1822 [4], *Triportheus guentheri* Garman, 1890, *Tetragonopterus chalceus* Spix & Agassiz, 1829 [5], *Brachyplatystoma vaillantii* Valenciennes, 1840 [6], *Cichla temensis* Humboldt, 1821 [7,8], and *Aequidens plagiozonatus* Kullander, 1984 [9], all in Brazil.

In Brazil, it is estimated that there are 2587 species of exclusively freshwater fish and 1297 salt water fish [10]. However, little is known regarding the diversity of protozoan parasites in these hosts in the country [11,12], such that for parasites of the phylum Apicomplexa, there are approximately 12 records distributed among haemogregarines and coccidia *stricto sensu*. Therefore, the purpose of this study was to determine the occurrence of *Calyptospora* in fish from a floodplain in the eastern Amazon.

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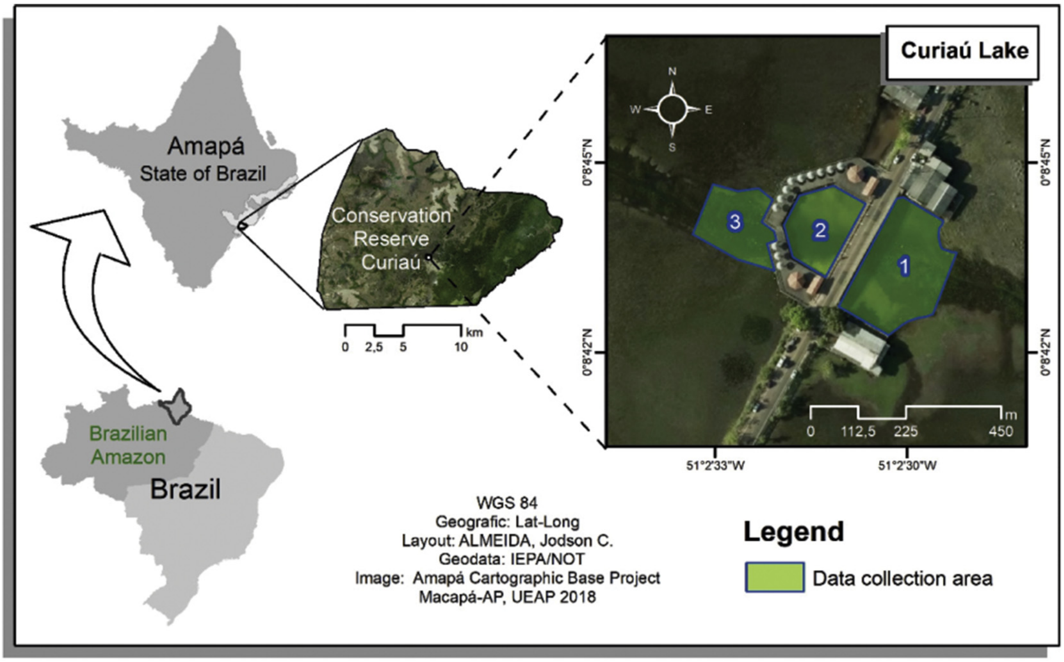

Fig. 1. Location of the samples in the Area of Environmental Protection of the River Curiaú, Macapá, Brazil.

2. Materials and methods

2.1. Study area

This study was conducted on floodplain of the Curiaú River, within the environmental protection area of the Curiaú (APA Curiaú), an area of 23,000 he, located in the municipality of Macapá, in the State of Amapá, Brazil (0°08′43.1″N; −51°02′29.2″W; 20°08′44.3″N; −51°02′33.4″W; and 3: 0°14′55.79″N, −51°04′24.16″W (Fig. 1). The location includes Amazonian savanna, terra firme and swamp forests, and seasonally flooded grasslands, as well as temporary and permanent lentic bodies of water [13]. The region has a very marked seasonality, with the dry season extending from July to December and rainy season from January to June. The highest rainfall occurs approximately from May to June [14].

2.2. Capture and examination of the fishes

The fish were captured by the team of the Laboratory of Morphophysiology and Animal Health (LABMORSA) of the State University of Amapá (UEAP), assisted by local fishermen using ~~of~~ 30 mm casting nets. The collection period was from June 2017 to January 2018 and 130 specimens were captured belonging to 12 different species. The fish were stored alive in thermal boxes with artificial aeration and transported to the LABMORSA, where they were housed in aquaria until the analyses were performed. The fishes were anesthetised with MS-222 (Sandoz Laboratories) at a concentration of 50 mg/ml. Next, the fishes were euthanised by spinal cord transection and fresh squash preparations of the liver, heart and gallbladder were examined under light microscope to determine the presence of *Calyptospora* spp.

2.3. DNA extraction and sequencing

Molecular procedures were performed only for the species *Hoplerythrinus unitaeniatus* Spix and Agassiz, 1829, *Geophagus proximus* Castelnau, 1855, and *Hoplias malabaricus* Bloch, 1794, from which liver fragments with oocysts were collected and stored in 80% ethyl alcohol. DNA was extracted from tissue samples using ReliaPrep gDNA Tissue Miniprep System (Promega), following the manufacturer's instructions.

The presence of parasites was initially determined using PCR with *Calyptospora*-specific primers Cal1F and 18R, following the parameters described by Whipps et al. [15]. However, after sequencing, amplification of the DNA of the host *G. proximus* was verified. A new primer pair was designed based on the sequences of *Calyptospora* spp. (GU479670, FJ904836, FJ904637, FJ904643, FJ904644, FJ904638, FJ904642), available in GenBank®, using GENEIOUS v.7.1.3 software (Biomatters; http://www.geneious.com). The primers mrF (forward, 5′-GGC-CGG-CGA-TAG-ATC-ATT-CA-3′, located at the position 299–318) and mrR (reverse, 5′-CGA-CAC-GCA-AAG-TCC-CTC-TA-3′, located at the position 1467–1486) amplify a fragment of approximately 1187 bp of the 18S rRNA gene of *Calyptospora* spp.

The PCR protocol involved 20 μl of reaction mixture containing 10.0 μl of GoTaq® Green Master Mix, 2 ×, 0.6 μl of dimethyl sulfoxide (DMSO), 1.2 μl of 25 mM $MgCl_2$, 0.6 μl of each primer, 1 μl of template DNA, and 6.0 μl of MilliQ water. Amplification was performed with initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR and sequencing reactions were performed by Helixxa Genomic Services (Paulínia, SP, Brazil). The amplified fragments were purified from the agarose gel and sequenced using the Applied Biosystems Big Dye v.3.1 kit with an ABI 3500 automatic sequencer (Applied Biosystems). Sequencing was performed in both directions.

2.4. Sequence alignment and phylogenetic analysis

The DNA sequences obtained were edited using the BioEdit software v7.2.5 [16] and compared for similarity with sequences available in GenBank® using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST). Multiple alignment, with 891 bp, for two sequences obtained in this study and 23 sequences retrieved from GenBank®, was performed using the MUSCLE algorithm and the GENEIOUS v.7.1.3 software (Biomatters; http://www.geneious.com). The jModelTest v.2.1.10 programme [17] was used to identify the best evolutionary model for maximum likelihood analysis. The best fitting model based on the Akaike Information Criterion (AIC) was TIM3 + I + G. The phylogenetic tree was inferred using Maximum Likelihood methods in the PhyML v.3.0 [18]. To estimate internal nodes of the tree, a bootstrap with 1000 replicates was used. An alignment with 870 bp, from two *Calyptospora* spp. sequences

Table 1
Prevalence of *Calyptospora* spp., feeding habits, and site of infection in fishes from the Area of Environmental Protection of the River Curiaú, Macapá, Brazil.

Species	Feeding habitats[#]	N	Prevalence (%)	Site of infection[*]
Crenicichla lepidota Heckel 1840	Carn	10	100	Lv
Cichla ocelaris Bloch and Schneider 1801	Carn	8	100	Lv
Hoplias malabaricus (Bloch 1794)	Omni	6	100	Lv
Mesonauta festivus (Heckel 1840)	Omni	6	100	Lv, Gb
Hoplerythrinus unitaeniatus (Spix and Agassiz 1829)	Omni	5	100	Lv
Astronotus ocellatus (Agassiz, 1831)	Carn	2	100	Lv, Gb
Geophagus proximus (Castelnau 1855)	Omni	12	75	Lv, Gb, He
Pterophyllum scalare (Schultze 1823)	Omni	19	63	Lv, Gb
Satanoperca jurupari (Heckel 1840)	Omni	22	54	Lv
Heros efasciatus Heckel 1840	Omni	7	28	Lv
Metynnis lippincottianus (Cope 1870)	Herb	21	0	–
Astyanax bimaculatus(Linnaeus 1758)	Herb	12	0	–
Total		130	55	

[*] Liver: Lv; Gallbladder: Gb; Heart: He.
[#] Herbivore: Herb; Carnivore: Carn; Omnivorous: Omni.

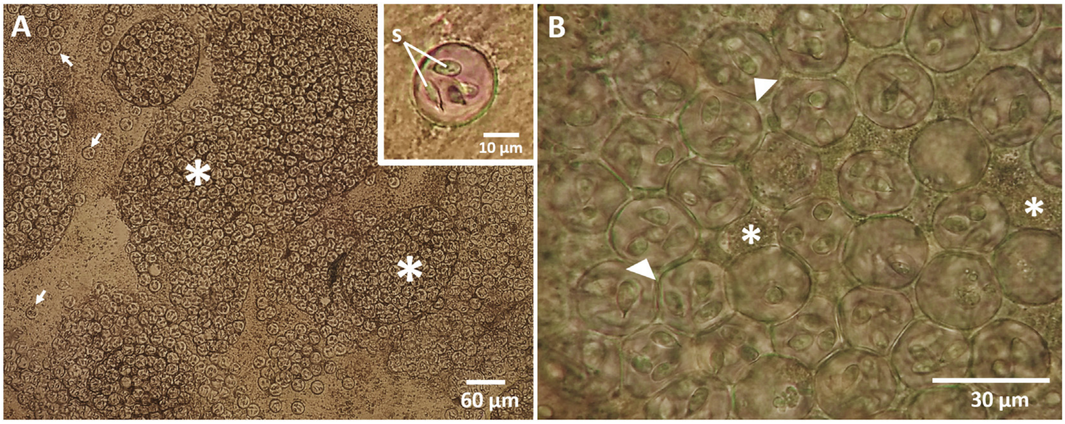

Fig. 2. *Calyptospora serrasalmi* from hepatic parenchyma of *Hoplias malabaricus*. (A) Individual oocysts of *C. serrasalmi* (arrows) and the formation of aggregates (*) s. sporocysts inside oocysts and (B) aggregates of oocysts containing four sporocysts (arrowheads) and unsporulated oocysts (*).

Table 2
Distance matrix among partial 18S rDNA sequences of *Calyptospora* spp. detected from fishes. Upper triangle shows the number of nucleotide difference, while the lower triangle shows the pairwise distance (*p* distance) among the sequences.

Sequences	1[*]	2[*]	3	4	5	6
1. *Calyptospora serrasalmi* (MH167351)[*]	–	0	2	36	42	34
2. *Calyptospora serrasalmi* (MH167352)[*]	0%	–	2	36	42	34
3. *Calyptospora serrasalmi* (FJ904639)	0.23%	0.23%	–	38	43	36
4. *Calyptospora spinosa* (FJ904636)	3.06%	3.06%	3.29%	–	62	63
5. *Calyptospora funduli* (FJ904644)	2.47%	2.47%	2.59%	4.23%	–	49
6. *Calyptospora paranaidji* (MH480605)	3.30%	3.30.%	3.53%	5.31%	4.23%	–

[*] Sequences from this study.

isolated in this study and four *Calyptospora* spp. sequences, detected from fishes available on GenBank®, was used to estimate *p*-distance on the MEGA6 software (Tamura et al., 2013).

3. Results

Calyptospora oocysts were observed in 55% of the 130 specimens and were present in 10 of the 12 species studied (Table 1). Parasite prevalence varied according to feeding habits, such that all the carnivorous species, with piscivorous tendencies, were positive, and 74% of the omnivorous species (invertivore and detritivore) presented infection. The herbivorous species *Astyanax bimaculatus* Linnaeus, 1758 and *Metynnis lippincottianus* Cope, 1870, which together accounted for 25% ($n = 33$) of the sample, were negative for infection.

All the infected specimens presented sporoblast oocysts and were completely sporulated (Fig. 2). Infection rates varied in relation to the viscera, where all the hosts presented parasitic forms in the liver, followed by 30% in the gallbladder and 9% in the heart. *Astronotus ocellatus* Agassiz, 1831, *Mesonauta festivus* Heckel, 1840, and *Pterophyllum scalare* Schultze, 1823, presented sporulated oocysts in the liver and gallbladder. In *G. proximus*, oocysts were verified in the liver, gallbladder, and heart. Livers with highest concentration of oocysts were swollen and showed lighter colouration than in organs and areas with lower concentrations of the parasites. Melano-macrophage centres were observed in specimens of *A. ocellatus*, *C. ocellaris*, *C. lepidota*, *H. unitaeniatus*, *H. malabaricus*, *M. festivus* and *P. scalare*, which had high infection intensity. *Geophagus proximus* sporulated oocysts were ovoid, 22.5 ± 1.5 µm in diameter, and piriform sporocysts were 9.0 ± 1.5 × 4.0 ± 0.6 µm, L/W 2.25. In *H. malabaricus*, sporulated oocysts were ovoid, 23.4 ± 1.5 µm in diameter, and piriform sporocysts were 8.2 ± 1.0 × 4.0 ± 0.4 µm, L/W 2.05.

Regarding the molecular characterisation of the parasites,

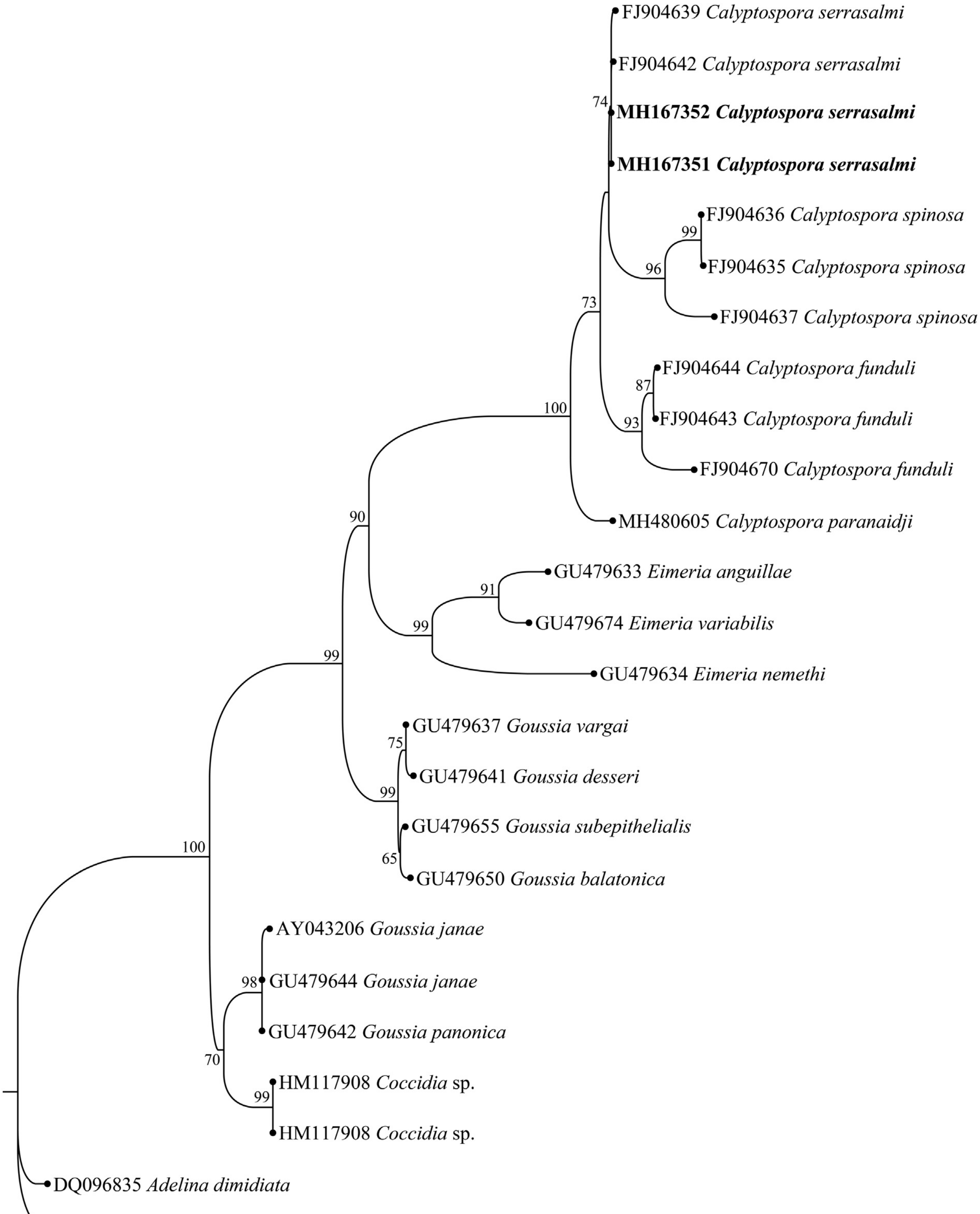

Fig. 3. Phylogenetic tree of maximum likelihood analysis based on the partial sequences (891 bp) of the 18S rRNA gene of *Calyptospora serrasalmi* from *Geophagus proximus* and *Hoplias malabaricus*, isolated in this study, and sequences of fish coccidian deposited in GenBank®. *Adelina dimidiata* and *Adelina grylli* were used as outgroups. The branch length scale represents 0.09 substitutions per site. The sequences obtained in this study are in bold.

unfortunately sequences were only obtained for *G. proximus* (MH167351) and *H. malabaricus* (MH167352). We verified 99% similarity to *C. serrasalmi* (FJ904642, FJ904639), isolated from *Serrasalmus rhombeus* Linnaeus, 1766, in Brazil, and 97% identity to *C. funduli* (FJ904643) from *Fundulus grandis* Baird and Girard, 1853, and *C. spinosa* (FJ904636) from *C. lepidota,* in the USA and Brazil, respectively. The *p*-distance among the *Calyptospora* sequences from this study and *C. serrasalmi* (FJ904639) was 0.24%, and among the *Calyptospora* sequences isolated herein and other *Calyptospora* species isolated from fishes, it ranged from 2.47 to 3.30% (Table 2). Based on the similarity search and nucleotide divergence (*p*-distance), we identified the sequences as a haplotype of *C. serrasalmi.* A phylogenetic tree inferred from a 891 bp fragment of the 18S rRNA gene (Fig. 3) showed that the *C. serrasalmi* sequences obtained in this study grouped within of a clade composed of *Calyptospora* spp. sequences and in the same branch as *C. serrasalmi* (FJ904642) isolated from *S. rhombeus*, with 74% bootstrap support.

4. Discussion

In this study, the infection rates of *Calyptospora* spp. varied according to the type of feeding habits of the fishes, with the highest number of infections in carnivorous fishes with a piscivorous tendency, such as *C. ocellaris*, *C. lepidota*, *H. unitaeniatus* and *H. malabaricus*, in comparison with omnivorous and herbivorous fishes. This is reinforced by the absence of infections in *A. bimaculatus* and *M. lippincottianus*, both mainly herbivorous [19–22]. In the life cycle of *Calyptospora*, fish infection occurs through the ingestion of shrimp, and the latter become infected by ingesting the remains of dead fish viscera containing sporulated oocysts [23]. Attempts to achieve experimental infection of fish by direct ingestion of *C. funduli* oocysts or infected viscera were not successful [24]. Based on the findings of this study, we recommend that new experiments are performed on carnivorous fish to verify the transmission of *Calyptospora* by predation.

Infected larger fish of the species *A. ocellatus*, *C. ocellaris*, *C. lepidota*, *G. proximus*, *H. unitaeniatus*, *H. malabaricus*, *M. festivus* and *Satanoperca jurupari* (Heckel 1840) presented *Calyptospora* oocysts in an extensive area of the hepatic parenchyma. Pathological changes, such as swelling and whitish appearance of the pancreas and liver, appear relatively common in *Calyptospora* infections, as reported for *A. gigas* [2] and *F. grandis* [24]. Similar observations were described by Eiras [25], Velasco et al. [8], Albuquerque and Brasil-Sato [5], Azevedo et al. [26] and Békési and Molnár [27]. Interestingly, despite the macro and microscopic damage observed in *Calyptospora* infection, inflammatory processes have not been recorded in fish infections [4,7].

The coccidian *C. serrasalmi* has been described by Cheung et al. [28] in piranhas *S. niger* of the northern region of Brazil, and the same parasite was later recorded in *Serrasalmus striolatus* Steindachner, 1908 in the north and northeast [15]. Although there is no record of *S. niger* in the floodplain of the Riu Curiaú, three other species of the genus occur in the region, *Serrasalmus calmoni* Steindachner, 1908, *S. rhombeus*, and *Serrasalmus spilopleura* Kner, 1858, and a species of *Serrasalmus* not yet identified [29]. It is very likely that these species participate in the cycles of *C. serrasalmi* at the collection site described above.

The identity of the invertebrate hosts of *Calyptospora* spp. in the region studied here is still unknown, but one or more species of shrimp may be involved in the cycles of transmission. Seven species of decapods occur at the study site, all belonging to the family Palaemonidae: *Palaemonetes carteri* Gordon, 1935, *Macrobrachium jelskii* Miers, 1877, *Euryrhynchus burchelli* Calman, 1907, *Euryrhynchus amazoniensis* Tiefenbacher, 1978, *Euryrhnchus wrzesniowskii* Miers, 1877, *Macrobrachium amazonicum* Heller, 1862, and *Pseudopalaemon cf. chryseus* Kensley and Walker, 1982 [30]. Since the *C. funduli* cycle involves the *Palaemonetes pugio* Holthius,1949 shrimp in the United States, it is highly probable that *P. carteri* participates in the cycles in the floodplain of the Riu Curiaú.

The recording of *C. serrasalmi* in two fish families, *G. proximus* (Cichlidae) and *H. malabaricus* (Erythrinidae), reinforces the idea that *Calyptospora* have low specificity in relation to their vertebrate hosts. Fournie and Overstreet [31] reported the successful experimental infection of *C. funduli* by ingestion of *P. pugio* shrimps in fish from five different families. In addition, *C. funduli* was recorded in *Opsanus beta* Goode and Bean, 1880 belonging to a sixth family, Batrachoididae [32].

5. Conclusion

In conclusion, *Calyptospora* spp. was microscopically identified in ten new species of fish, and in 2 of them the identification was confirmed at molecular level.

Compliance with ethical standards

The capture of fishes was authorised by the Amapá State Secretary for the Environment, Brazil (authorisation no. 1014/2016) and by Chico Mendes Institute for Biodiversity Conservation (ICMBio), under authorisation no. 50376-1/2017, based on the pertinent Brazilian legislation. Procedures with the fishes were approved by Animal Ethics Committee of EMBRAPA/CPAA, under authorisation no. 21157.001846/2018-91.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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