First report of Crassiphiala sp. (Trematoda: Diplostomidae) as an etiological agent of black spot disease in commercial ornamental fish from Brazil

Danimar López-Hernández
Federal University of Minas Gerais
Marcia Pimenta Leibowitz
Federal University of Minas Gerais
Hudson Alves Pinto
Federal University of Minas Gerais
Carlos Augusto Gomes Leal (carlosleal@vet.ufmg.br)
Federal University of Minas Gerais

Short Report

Keywords: Molecular phylogeny, black spot fish-eating birds disease, fish, trematodes

Posted Date: September 27th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2088593/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Parasitology Research on February 17th, 2023. See the published version at https://doi.org/10.1007/s00436-023-07794-7.

Abstract

Ornamental fish are becoming increasingly popular, but the lack of knowledge regarding their various diseases is a major challenge. Skin diseases commonly found in freshwater fish include black spot disease (BSD), which is characterized by melanin deposits around the metacercariae of some trematode species. Since BSD remains poorly understood, this study describes an outbreak of BSD in *Etroplus maculatus* raised in outdoor ponds at a Brazilian fish farm. Metacercariae samples were collected, examined, and subjected to molecular phylogenetic analysis. The parasites were conspecific to an unnamed species, *Crassiphiala* lineage 5, recently found in Brazilian birds (*Megaceryle torquata*). Sequences obtained for longifurcate cercariae of the planorbid snail *Biomphalaria straminea* from the same region were identical to our metacercariae of *Crassiphiala* sp. These results suggest that *Biompahalaria* snails are likely an intermediate host of this parasite on farms where *E. maculatus* was found to be infected. We provide the first molecular evidence that *Crassiphiala* are the causative agents of BSD in fish from Brazil. Combatting snails and preventing access of fish-eating birds to outdoor ponds are strategies to control this disease in ornamental fish farms.

1. Introduction

The aquaculture industry in Brazil focuses mainly on fish products for human consumption. However, the pet industry is growing steadily, and ornamental fish are now the fourth most popular pet in the country Rezende et al., 2021; Valenti et al., 2021;). Freshwater ornamental fish farming occurs mainly in southeastern Brazil, especially in the states of São Paulo and Minas Gerais (Faria, 2016; Valenti et al., 2021).

Among the diseases commonly reported in freshwater fish are black spot disease (BSD), which is mainly caused by parasitism by members of the family Diplostomidae (Lane and Morris, 2000; Niewiadomska, 2002), but also by some species of Heterophyidae (Sándor et al., 2017; Denis et al., 2019; Duflot et al., 2021). The name of this disease alludes to the macroscopic aspect of infected animals, where melanin produced by melanomacrophages is deposited around the encysted metacercariae. (Bush et al., 2001; Thatcher, 2006; Denis et al., 2019). This alteration also results in an unpleasant appearance of fish reared for ornamental purposes and contributes to rejection by consumers, which can be detrimental to businesses (Lane and Morris, 2000).

We aimed to identify the etiological agent of BSD in *Etroplus maculatus*, a farmed ornamental fish species in Brazil.

2. Material And Methods

2.1. Fish sampling and parasitological examinations

An outbreak of BSD was observed in *E. maculatus* raised in outdoor tanks at a Brazilian fish farm. A total of 13 live fish (mean weight of 3 g) were collected and immediately transported to diagnostic laboratory. Fish were kept in aquaria, fed commercial fish food, and processed for parasitological analysis.

2.2. Parasitological examinations

For metacercariae recovery, the fish were euthanized by immersion in a benzocaine solution (250 mg/L). The fish integuments (skin and fins) were examined under a stereomicroscope, the metacercariae were counted, and cysts were removed using metal needles. These were mounted between glass and coverslips. Larvae were mechanically excystized and mounted as described above or killed in 70 ºC water, and fixed in 10% formalin. Specimens were stained with alum acetocarmine, dehydrated in a crescent ethanol series, diaphanized in beechwood creosote, and mounted between slides and coverslips with Canada balsam. Parasite preparations were examined under a light microscope (Leica DM500) and photographed using a Leica ICC50 HD digital camera. The dimensions of the cyst and larvae were obtained from the photographs and represented in micrometers (µm).

We attempted to obtain the adult stage of the parasite for specific taxonomic identification by orally inoculating four young chickens (*Gallus dosmeticus*) and two jirds (*Meriones unguiculatus*). They were forced to swallow a solution containing 20 encysted metacercariae removed from *E. maculata*. The vertebrates were kept under laboratory conditions with ad libitum access to water and food. Fecal samples were examined using the spontaneous sedimentation technique to evaluate infection success.

Animals were euthanized and necropsied 12 days post-infection to detect adult parasites in their intestines. The use of vertebrate animals in the experiments followed the protocol approved by the Local Ethics Committee in Animal Experimentation (CEUA-UFMG, protocol 68/2017).

2.3 Molecular analyses

Samples of encysted metacercariae were fixed in ethanol 95% and stored at -20°C until use. Partial regions of the 28S (primers Dig12 and 1500R), ITS (primers D1 and D2), and c*ox*1 (primers JB3 and COI-R Trema) genes were amplified by PCR, using previously described conditions (Galazzo et al., 2002; Tkach et al., 2003; Miura et al., 2005). Additionally, cercariae previously found in *Biomphalaria straminea* in the same geographic area (Lopez-Hernandez et al., 2019) were sequenced using the same region of *cox*1 to evaluate the similarity to *E. maculatus*.

Sequence data were edited using Chromas Pro software (Technelysium Pty Ltd, Australia), and the contigs were used for similarity searches in the Basic Alignment Search Tool (BLAST). Alignments were constructed using MEGA X (Kumar et al., 2018), and sequences of the closest genera available in GenBank were included. The evolutionary models used in the phylogenetic analysis were determined using the Bayesian information criterion in MEGA X and outgroup selections were based on phylogenies published by Achatz et al. (2019). Phylogenetic analyses were performed using maximum likelihood and Bayesian inference methods. The maximum likelihood trees were generated in MEGA X (bootstrap test with 1000 repetitions). Bayesian inference analyses were performed in MrBayes v.3.2.6 (Ronquist et al.,

2012) using Markov Monte Carlo chain searches in two simultaneous runs of four chains per 1,000,000 generations and sampling every 100 generations. The first 25% of the examined trees were discarded as 'burn-in'.

3. Results And Discussion

Morphological and molecular data on metacercariae involved in BSD in *E. maculatus* revealed the presence of a species of the genus *Crassiphiala* Van Haitsma, 1925 (Diplostomidae: Crassiphialinae). A total of 144 metacercariae were counted in the 13 specimens evaluated, with a mean infection intensity of 11 ± 8 (3–32) metacercariae/fish. Macroscopically, cysts were found in the teguments and fins (Fig. 1), and they were covered by a dark melanin deposit produced by melanomacrophages. The resulting black spots were 0.5 mm in size (Fig. 2 A, B).

Morphologically, the cysts were small, oval to spherical, measuring 337 ± 19 (310–366) µm × 339 ± 27 (295–383) µm, with a resistant wall, 33 ± 8 (26–47) µm thick, containing an oval internal cyst, 218 ± 6 (212–224) µm thick, with a thin tick membrane (Fig. 2C). Excysted larvae were very small, with a body bipartite, 409 ± 71 (303–454) µm (Figure 2D, E). The forebody was elongated to oval, 216 ± 37 (163–245) µm × 101 ± 16 (89–125) µm. The hind body was oval, 194 ± 35 (141–219) µm × 149 ± 12 (135–163) µm. The oral sucker was subterminal with an oval shape of 36 ± 3 (32–38) µm × 36 ± 5 (29–41) µm. The pharynx muscle was spherical, measuring 25 ± 3 (22–28) µm × 26 ± 2 (24–29) µm. The ventral sucker was transversely oval, measuring 33 × 45 µm. The holdfast organ was transversely oval, occupying 50% of the forebody, which was a differential trait related to the species of *Crassiphiala*. Attempts to obtain adults from vertebrates for morphological identification were unsuccessful.

Sequences of 28S (1227 bp), ITS (1200 bp), and mitochondrial c*ox*1 (799 bp) were obtained for *E. maculatus* metacercariae. High identity with *Crassiphiala* was verified, 97.6–99.8% using the 28S gene. Phylogenetic analysis of 28S and ITS sequences revealed that the parasite grouped in well-supported clades containing *Crassiphiala* spp. (Fig 3). Based on 28S, 100% similarity was found with *Crassiphiala* sp. lineage 5 (MN200261). This sequence information was recovered from *Megaceryle torquata* in the Brazilian Pantanal (Achatz et al., 2019). Based on ITS, 100% similarity was verified for cercariae found in *Biomphalaria straminea* in Belo Horizonte, Brazil (MN179277).

Analysis of c*ox*1 resulted in a trimmed alignment containing 372 bp and two species/lineages of the genus *Crassiphiala*. New sequences were also obtained for the same region of c*ox*1 in a longifurcated cercaria (Strigeid cercaria) previously reported in *B. straminea* from the same geographical area (López-Hernández et al., 2019). Phylogenetic analysis revealed both larval isolates were from the same clade as *Crassiphiala* sp. lineage 5 (Achatz et al., 2019). The combination of this information suggests that the isolates of *Crassiphiala* are conspecific. However, the species differs from *Crassiphiala* sp. lineage 2 (molecular similarity: 92.6–93.6%). Comparison of *cox*1 sequences of the barcode region (for cercariae from *B. straminea*) confirmed the link with *Crassiphiala* sp. lineage 5 (96.8–97.0% similarity with *M.*

torquata isolates). Relatively low similarities (82.7–87.7%) were found with the other four lineages/species of *Crassiphiala.*

The involvement of diplostomid species in fish BSD around different parts of the world is well known (Thatcher 2006; Flores-Lopes 2014; Barrilli et al., 2021). However, no studies have identified the etiological agent and other hosts involved in the parasitic life cycle (Denis et al., 2019; Kohl et al., 2019; Charo-Karisa et al., 2021; Duft et al., 2021). Our data suggest that an unnamed species linked to *Crassiphiala* sp. lineage 5 is involved in *E. maculata* BSD. The present study is the first report of BSD caused by a *Crassiphiala* species in a farmed ornamental fish.

Species of *Crassiphiala* are intestinal parasites of kingfishers in the adult stage (Achatz et al., 2019), and are identified here as causative agents of BSD. This disease is characterized by parasitic invasion and encysting in various fish organs, usually the skin and muscle, where they are externally visible as black spots (Hoffman 1955; Achatz et al. 2019). The complete life cycle is known only for *Crassiphiala bulboglossa*. Adult parasites were found in kingfishers, and neascus-like melanized metacercariae were found encysted in the fish skin (Hoffman, 1956).

The genus *Crassiphiala* was only recently discovered in South America after two molecular lineages/species were found in kingfishers (*M. torquata*) (Achatz et al.2019). Specific identification of this parasite requires detailed morphological characterization of the adult stages for a formal taxonomic description as a new species of *Crassiphiala*. We attempted to obtain the adult stage, but no worms were found in the young chickens tested. Future helminthological studies are required to identify the adult stage of the parasite in *E. maculatus*.

Our study identified larval stages that were previously found in *B. straminea* from the same geographical area, and identified them as *Crassiphialinae* gen. sp. (López-Hernández et al., 2019); they were molecularly linked to the metacercariae found in *E. maculatus*. This planorbid snail is widely distributed and adapted to human-made aquatic environments. Their presence in outdoor ponds represents a risk for BSD and other fish diseases caused by trematodes that present piscivorous birds as definitive hosts. Field studies on fish farms are needed to definitively determine this snail as a vector of *Crassiphiala*.

Declarations

Competing interest statement

The authors declare no competing financial interests.

Acknowledgments

This work was supported in part by the National Council for Scientific and Technological Development (CNPq; research scholarship to HAP].

Funding statement

This work was supported in part by the National Council for Scientific and Technological Development (CNPq; research scholarship to HAP).

Conflict of interest disclosure

The authors declare no competing financial interests.

Availability of data and material

Data available upon reasonable request from the first author or the corresponding author.

Authors' contributions

1- Danimar López-Hernández: acquisition of data, performed the experiments, analysis and interpretation of results and for drafting the article.

2-Marcia Pimenta Leibovitz: analysis and interpretation of results, drafting the article, and revising it critically for important intellectual content

3- Hudson Alves Pinto: conception and design of the study, analysis and interpretation of results, revising it critically for important intellectual content, and final approval of the version to be submitted.

4-Carlos Augusto Gomes Leal: conception and design of the study, supervision of the study, funding acquisition, revising it critically for important intellectual content, and final approval of the version to be submitted.

Ethics approval statement

The study was approved by the Local Ethics Committee in Animal Experimentation (CEUA-UFMG, protocol 68/2017).

Consent statement to participate

This is not applicable to the present manuscript.

Consent statement for publication

All authors reviewed and approved final version of the manuscript.

References

1. Achatz, TJ, Pulis, EE, Fecchio, A, Schlosser, IJ, Tkach, VV (2019) Phylogenetic relationships expanded diversity and distribution of *Crassiphiala* spp. (Digenea, Diplostomidae), agents of black spot disease in fish. Parasitol Res 118:2781-2787. https://doi.org/10.1007/s00436-019-06439-y

2. Barrilli, GHC, Moura, ENF, Rocha, O, Verani, JR (2021) The influence of black spot disease on the relative condition factor of *Astyanax paranae* Eigenmann, 1914 (Characiformes: Characidae) in Brazilian subtropical streams. Acta Sci Biol Sci 43:e56164. https://doi.org/10.4025/actascibiolsci.v43i1.56164
3. Bush, AO, Fernandez, JC, Esch, GW, Seed, JR (2001) Parasitism: The diversity and ecology of animal parasites. Cambridge: United Kingdom.
4. Charo-Karisa, H, Ali, SE, Marijani, E, Ibrahim, NA, Trinh, TQ, Chadag, MV, Benzie, JAH (2021) Genetic parameters for black spot disease (diplopstomiasis) caused by *Uvulifer* sp. infection in Nile tilapia (*Oreochromis niloticus* L.) Aquac 532:736039.
5. Dennis, MM, Izquierdo, A, Conan, A, Johnson, K, Giardi, S, Frye, P, Freeman, MA (2019) Scaphanocephalus-associated dermatitis as the basis for black spot disease in Acanthuridae of St. Kitts, West Indies. Dis Aquat Org 137:53-63. https://doi.org/10.3354/dao03419
6. Duflot, M, Gay, M, Midelet, G, Kania, PW, Buchmann, K (2021) Morphological and molecular identification of *Cryptocotyle lingua* metacercariae isolated from Atlantic cod (*Gadus morhua*) from Danish seas and whiting (*Merlangius merlangus*) from the English Channel. Parasitol Res 120:3417-3427. https://doi.org/10.1007/s00436-021-07278-6
7. Flores-Lopes, F (2014) The occurrence of black spot disease in *Astyanax* aff. *fasciatus* (Characiformes: Characidae) in the Guaíba Lake basin, RS, Brazil. Braz J Biol 74:S127-34. https://doi.org/10.1590/1519-6984.08312
8. Galazzo, DE, Dayanandan, S, Marcogliese, DJ, McLaughlin, JD (2002) Molecular systematics of some North American species of *Diplostomum* (Digenea) based on rDNA-sequence data and comparisons with European congeners. Can J Zool 80:2207-2217. https://doi.org/10.1139/z02-198
9. Kohl, ZF, Calhoun, DM, Elmer, F, Peachey, RBJ, Leslie, KL, Tkach, V, Kinsella, JM, Johnson, PTJ (2019) Black-spot syndrome in Caribbean fishes linked to trematode parasite infection (*Scaphanocephalus expansus*). Coral Reefs 38:917-930. https://doi.org/10.1007/s00338-019-01819-3
10. Kumar, S, Stecher, G, Tamura, K. (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870-1874. https://doi.org/10.1093/molbev/msw054
11. Lane, RL, Morris, JE (2000) Biology, prevention, and effects of common grubs (digenetic trematodes) in freshwater fish. NCRAC Technical Bulletins 115:1–6.
12. López-Hernández, D, Locke, AS. Assis, JCA, Drago, F, Melo, AL, Rabelo, ÉM, Pinto, HA (2019) Molecular, morphological and experimental-infection studies of cercariae of five species in the superfamily Diplostomoidea (Trematoda: Digenea) infecting *Biomphalaria straminea* (Mollusca: Planorbidae) in Brazil. Acta Trop 199:105082. https://doi.org/10.1016/j.actatropica.2019.105082
13. Miura, O, Kuris, AM, Torchin, ME, Hechinger, RF, Dunham, EJ, Chiba, S (2005) Molecular-genetic analyses reveal cryptic species of trematodes in the intertidal gastropod, *Batillaria cumingi* (Crosse). Int J of Parasitol 35:793-801. https://doi.org/10.1016/j.ijpara.2005.02.014
14. Niewiadomska, K (2002) Family Diplostomidae Poirier, 1886. In DI Gibson, A Jones, R.A (Eds.). Keys to the Trematoda. London, pp. 167-196

15. Ronquist, F, Teslenko, M, van der Mark, P, Ayres, DL, Hohna, S, Larget, B, Liu, L, Suchard, MA, Huelsenbeck, JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61:539-542. https://doi.org/10.1093/sysbio/sys029
16. Sándor, D, Molnár, K, Gibson, DI, Székely, C, Majoros, G, Cech, G (2017) An investigation of the host-specificity of metacercariae of species of Apophallus (Digenea: Heterophyidae) in freshwater fishes using morphological, experimental, and molecular methods. Parasitol Res 116:3065-3076. https://doi: 10.1007/s00436-017-5617-5
17. Schaaf, CJ, Kelson, SJ, Nusslé, SC, Carlson, SM (2017) Black spot infection in juvenile steelhead trout increases with stream temperature in northern California. Environ Biol Fishes 100:733–744. https://doi.org/10.1007/s10641-017-0599-9
18. Tkach, VV, Littlewood, DTJ, Olson, PD, Kinsella, JM, Swiderski, Z (2003) Molecular phylogenetic analysis of the Microphalloidea Ward, 1901 (Trematoda: Digenea). Syst Parasitol 56:1–15. https://doi.org/10.1023/A:1025546001611
19. Wanja, DW, Mbuthia, PG, Waruiru, RM, Bebora, LC, Ngowi, HA (2020) Natural Concurrent Infections with Black Spot Disease and Multiple Bacteriosis in Farmed Nile Tilapia in Central Kenya. Vet Med Int 2020:8821324. https://doi:10.1155/2020/8821324

Figures

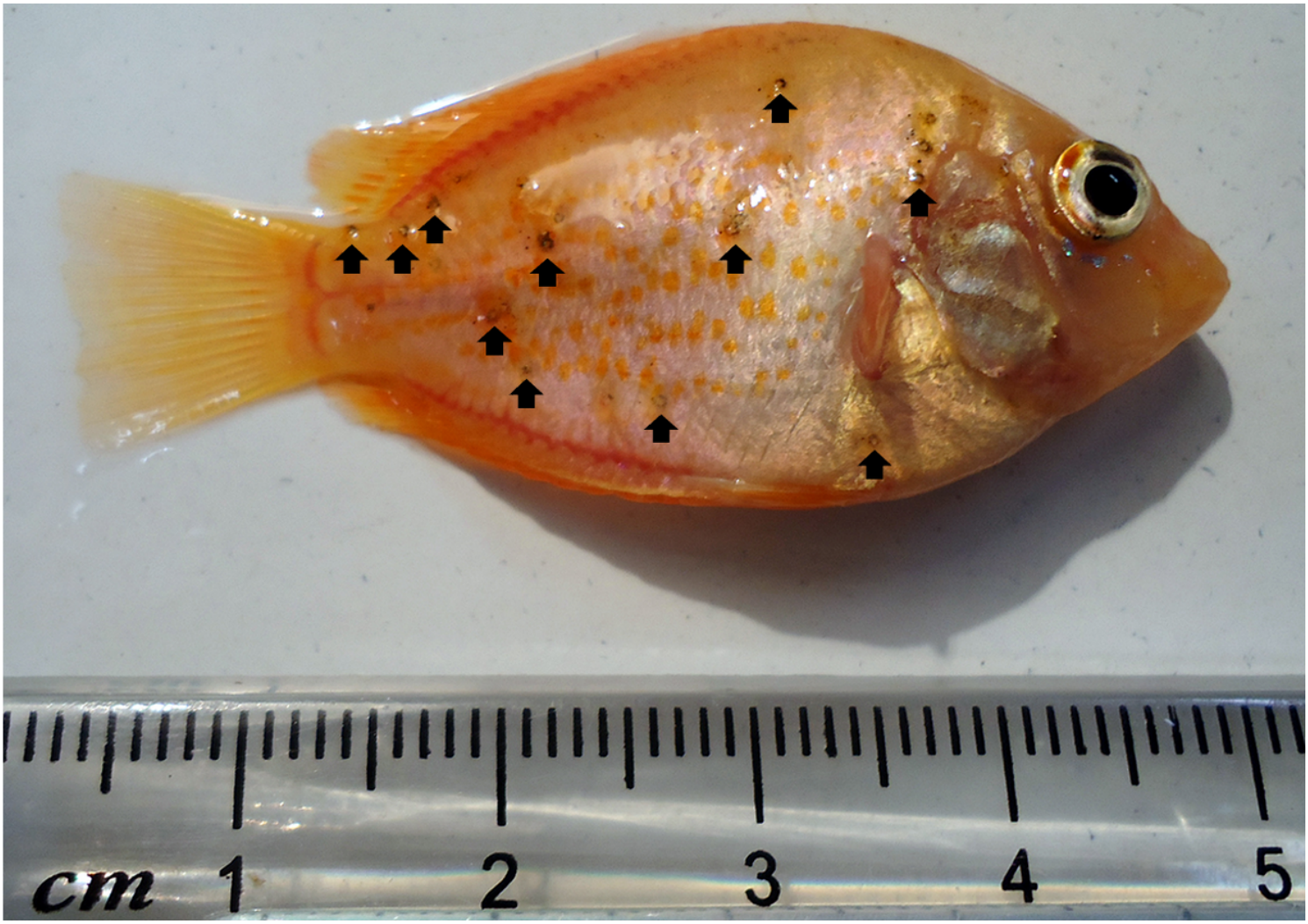

Figure 1

Specimen of mandarin fish,*Etroplus maculatus*, representing the “black spot disease” (arrows) caused by metacercariae of *Crassiphiala* sp.

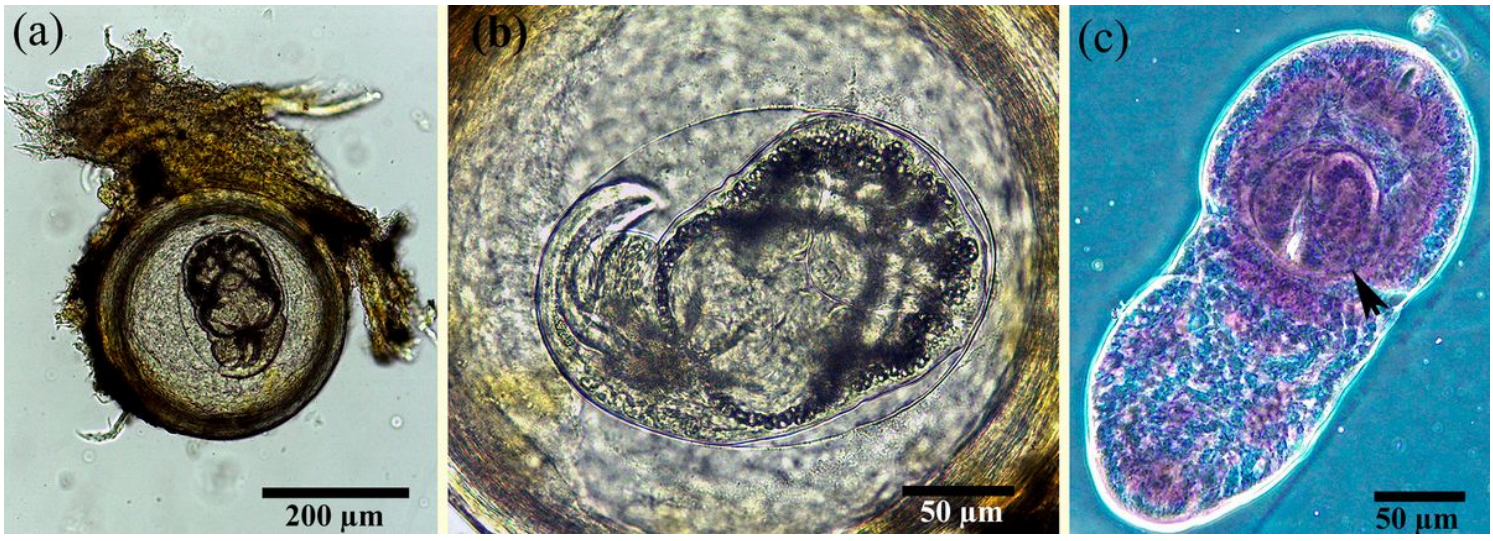

Figure 2

Metacercariae of *Crassiphiala* sp. found in mandarin fish, *Etroplus maculatus*. Encysted metacercaria (A). Detail of the larva inside the cyst (B). Excysted metacercaria alive (C) and after staining (D), showing the large tribocytic organ (arrow), a distinctive morphological trait of the genus.

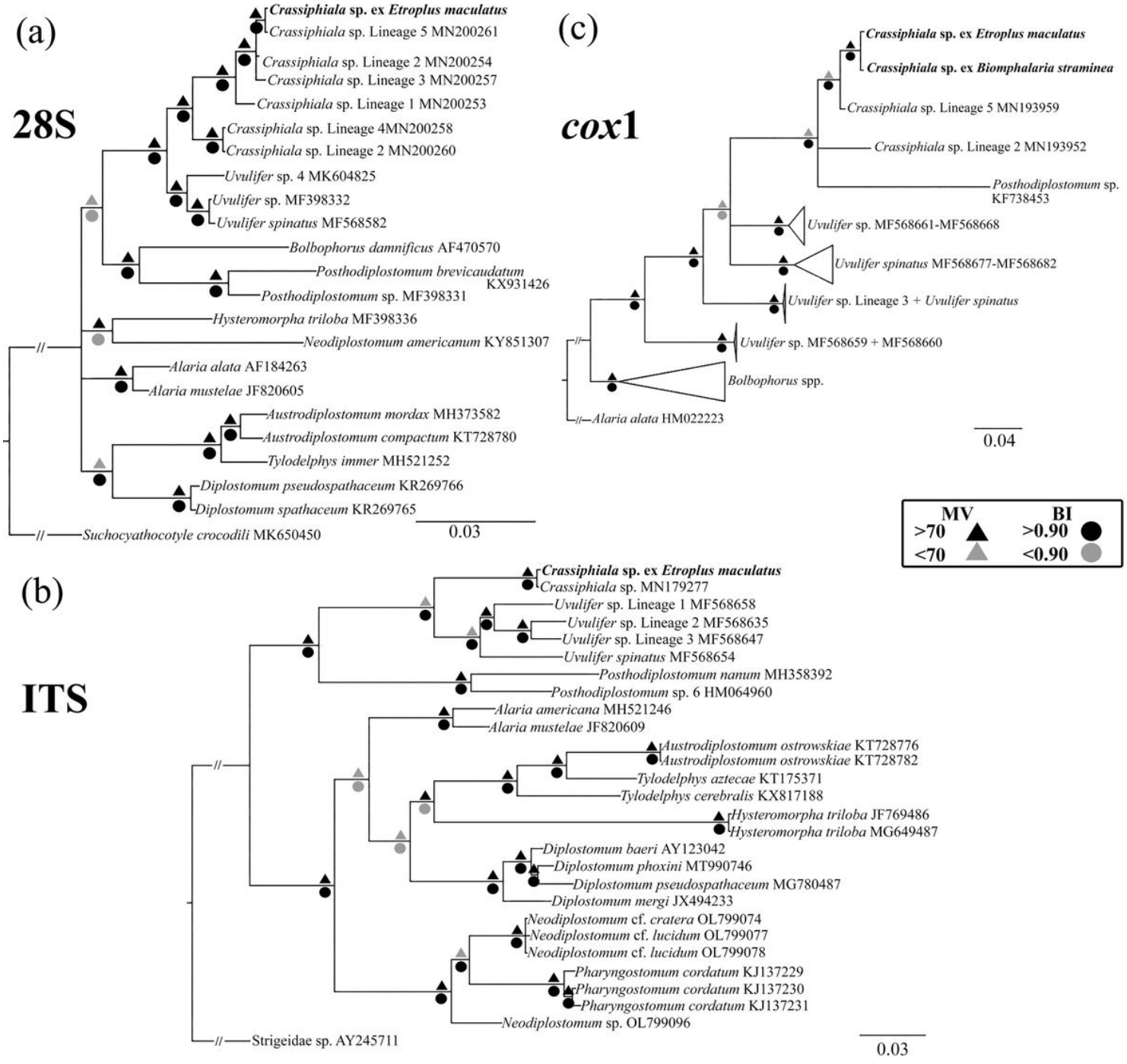

Figure 3

Phylogenetic relationships between *Crassiphiala* sp. (in bold) found in mandarin fish,*Etroplus maculatus*, and other species of Diplostomidae. Phylogenetic trees were inferred from sequences of genes 28S (A), ITS (B), and *cox*1 (C), which were analyzed by Bayesian inference and maximum likelihood methods.