Journal of King Saud University – Science 34 (2022) 101936

Contents lists available at ScienceDirect

Journal of King Saud University – Science

journal homepage: www.sciencedirect.com

Original article

Morphological and molecular phylogenetic analysis of *Bivesicula claviformis* Yamaguti, 1934 infecting the tomato hind *Cephalopholis sonnerati* (Serranidae) in Saudi Arabia

Jawahir Alghamdi [a], Saleh Al-Quraishy [a], Rewaida Abdel-Gaber [a,b,*]

[a] *Zoology Department, College of Science, King Saud University, Riyadh, Saudi Arabia*
[b] *Zoology Department, Faculty of Science, Cairo University, Cairo, Egypt*

ARTICLE INFO

Article history:
Received 19 January 2022
Revised 5 February 2022
Accepted 28 February 2022
Available online 2 March 2022

Keywords:
Host-specificity
Serranidae
Bivesiculata
Phylogeny

ABSTRACT

Groupers are large predators in the Serranidae family that feed on fish and small invertebrates. Identification of parasitic taxa of groupers was done concerning their morphological and biological characteristics. A total of 30 *Cephalopholis sonnerati* (Serranidae) specimens were investigated for digenetic trematodes in the Red Sea, Saudi Arabia. Only one bivesiculid species, belonging to the Bivesiculidae family, has been identified, with a prevalence rate of 60% (18/30) among infected fish. The *Bivesicula* species obtained from the fish host's intestine is morphologically and morphometric compatible with *Bivesicula claviformis*, which was previously identified from *Epinephelus fasciatus* (Serranidae) in North Borneo. In addition, maximum parsimony based on Tamura-Nei model was utilized to determine the phylogeny of the recovered *Bivesicula* species using partial small subunit ribosomal RNA gene (*18S rRNA*) and large subunit ribosomal RNA gene (*28S rRNA*) sequences. The query sequences showed the identities of 99.39% and 94.02% for *18S* (AJ287485.1) and *28S* rRNA (AY222182.1) of the previously described *Bivesicula claviformis*, respectively. The present study demonstrated that *Bivesicula claviformis* is the first reported of this genus as endoparasites from *C. sonnerati*, as well as providing novel DNA data for this species.

1. Introduction

The Red Sea and the Arabian Gulf are both accessible to Saudi Arabia. The Red Sea provides more than half of the country's marine fish (Shellem et al., 2021). The family Serranidae Swainson, 1839 sometimes known as groupers, is one of Saudi Arabia's most important food fish (Hariri et al., 2000). Groupers have a lot of biodiversities, and they are known to have various parasite fauna (Rohde and Heap, 1998). Identification for parasitic taxa of groupers associated with their morphological and biological peculiarities (Justine et al., 2010).

The Bivesiculidae Yamaguti, 1934 is a small family of trematodes that mostly infects the intestines of marine fish (Cribb, 2002; Trieu et al., 2015). Yamaguti (1934) established the genus *Bivesicula* for a single species, *Bivesicula claviformis*, as the first representative of Bivesiculidae. There are currently 27 accepted species from five recognized genera, *Bivesicula* Yamaguti, 1934, *Bivesiculoides* Yamaguti, 1938, *Treptodemus* Manter, 1961, *Paucivitellosus* Coil et al., 1965, and *Treptodemoides* Shen, 1995 (WoRMS, 2021). Bivesiculids have just two hosts in their life cycle, and the cercariae emerge from the snail and are consumed without encysting by the final fish host (Holocentridae, Muraenidae, and Serranidae) (Le Zotte, 1954; Pearson, 1968; Mani, 1989). Cribb et al. (1998) hypothesized that bivesiculids may have three host life cycles, due to the isolation of metacercaria of *Bivesicula claviformis* from the intestine of *Thalassoma lunare* from Lizard Island. The three host life cycles enable bivesiculids to infect large carnivorous fish (Trieu et al., 2015).

The genus *Bivesicula* is the most complicated genus within Bivesiculidae, with 16 recognized species and several undefined "types" represented by a single morphological specimen (WoRMS, 2021). The type-species of the genus, *Bivesicula claviformis*, is the most problematic. Because of the significant morphological resemblance across *Bivesicula* species, genetic evidence is needed to supplement traditional techniques of species classifica-

---

* Corresponding author.
E-mail address: rabdelgaber.c@ksu.edu.sa (R. Abdel-Gaber).

Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jksus.2022.101936
1018-3647/

tion. Recent research has shown that the ribosomal DNA (*rDNA*), which is made up of coding regions (*18S*, *5.8S*, and *28S*) as well as a non-coding region of two internal transcribed spacers (*ITS-1*, *ITS-2*) and one non-transcribed spacer (*NTS*), may be used to identify parasites (Wei et al., 2006).

There is no sufficient information about the infection with *Cephalopholis sonnerati*. Therefore, the study was designed to investigate more about the morphology and phylogeny of one bivesiculid in this fish species from the Red Sea in Saudi Arabia.

2. Materials and methods

2.1. Fish collection and parasitological study

During the period of January-September 2021, 30 fish specimens of the tomato hind *Cephalopholis sonnerati* (F: Serranidae) were bought from fish markets of the Red Sea coastlines in Jeddah, Saudi Arabia. A comprehensive examination of each fish was conducted to discover metazoan parasites using the gut-wash method (Cribb and Bray, 2010). Flukes were removed, washed in saline, and then preserved in a buffered formalin solution (10%) for morphological study, or kept immediately in 100% ethanol for DNA extraction. Worms were stained with Semichon's acetocarmine, cleared with clove oil, and mounted with Canada balsam for entire mounts. Using Leica DM 2500 microscope (NIS ELEMENTS software, ver. 3.8), photographs of each parasite species (along with comprehensive photos of diagnostic morphological features) were taken at different magnifications. Drawing of the recovered parasite specimens was done with the help of camera Lucida. According to Bush et al. (1997), the prevalence and mean intensity of parasite species were calculated. The range of measurements was given in millimeters (mean in parentheses).

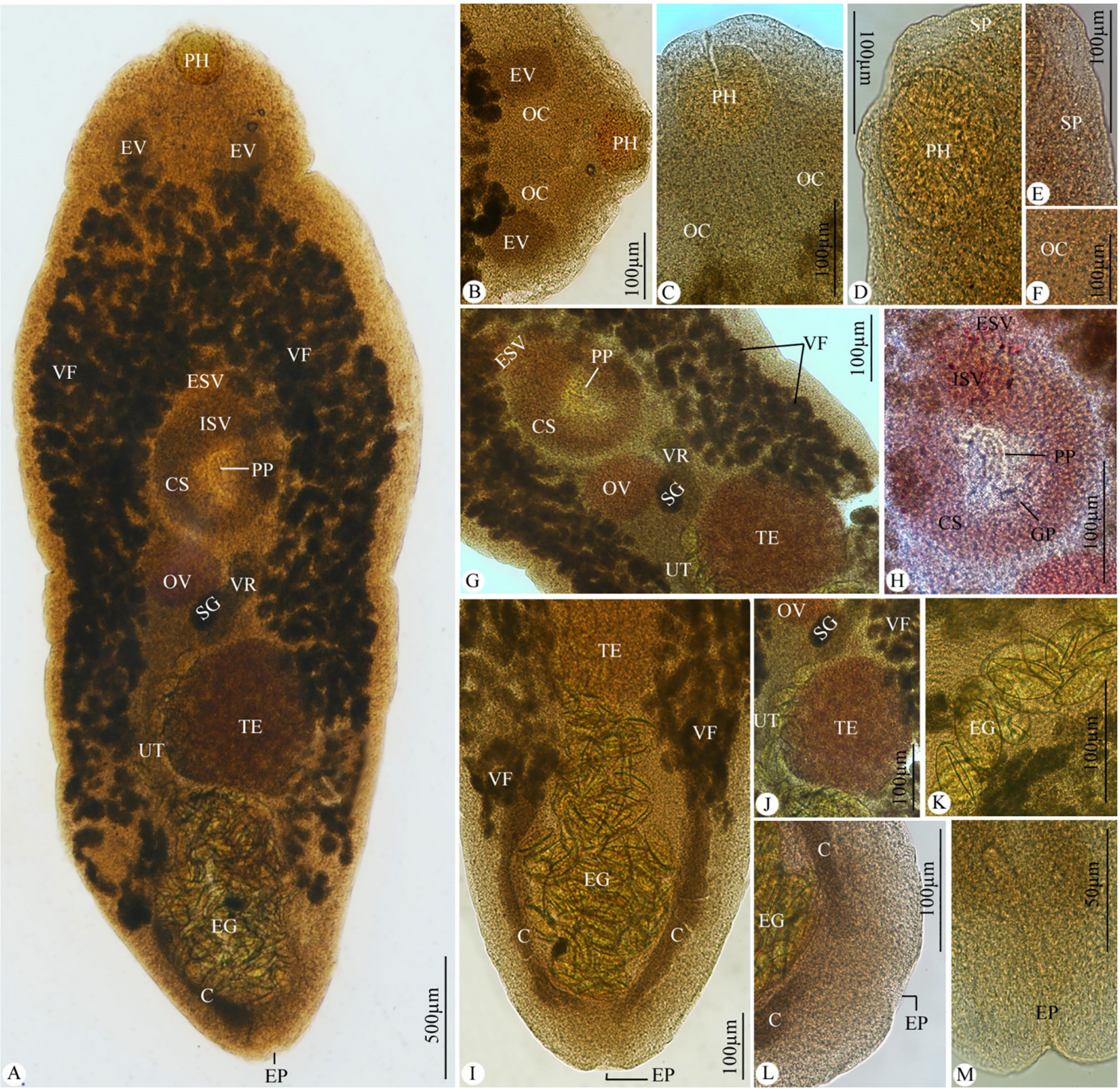


Fig. 1. Photomicrographs of *Bivesicula claviformis* infecting *Cephalopholis sonnerati*. (A) Whole-mount preparation. (B-F) Anterior portion of the body. (G, H) Middle portion of the body. (I-M) Posterior portion of the body. Note: C, ceca; CS, cirrus sac; EG, eggs; EP, excretory pore; ESV; external seminal vesicle; EV, excretory vesicle; GP, genital pore; ISV, internal seminal vesicle; OC, ocular region; OV, ovary; PH, pharynx; PP, pars prostatica; SG, shell gland; SP, spinules; TE, testis; UT, uterus; VF, vitelline follicles; VR, vitelline reservoir.

2.2. Molecular analysis

The genomic (g) DNA was extracted using the Qiagen DNeasy Tissue Kit© (Hilden, Germany) according to the steps of the manufacturer. For identification of the recovered parasite, partial *18S rRNA* and *28S rRNA* regions were amplified using polymerase chain reaction (PCR). Indaryanto et al. (2015) designed primers for *18S rRNA* PCR amplification that used in this study of 18SU467_F: 5′–ATC CAA GGA AGG CAG CAG GC–3′ and 18SL1170_R: 5′–GTG CCC TTC CGT CAA TTC CT–3′. Furthermore, Lee et al. (2007) designed primers that were used here for *28S rRNA* of JB10_F: 5′–GAT TAC CCG CTG AAC TTA AGC ATA–3′ and JB9_R: 5′–GCT GCA TTC ACA AAC ACC CCG ACT C–3′. The following thermocycling profile was used: 1 cycle of 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 62 and 66 °C (for *18S rRNA* and *28S rRNA*, respectively) for 30 s, 72 °C for 2 min, and 1 cycle of final extension at 72 °C for 7 min. QIAquick™ PCR Purification Kit (Qiagen, Hilden, Germany) was used to purified the PCR amplicons. The PCR products were sequenced (see details in Abdel-Gaber et al., 2020). Under accession numbers OK181883.1 and OK353794.1, nucleotide sequences were submitted in GenBank (NCBI, Bethesda, Maryland). To find the closest sequences, BLASTn compared the sequences in the NCBI database. The sequences were aligned using the BioEdit (Hall, 1999) and ClustalW multiple alignment software (Thompson et al., 1994). MEGA 7.0 was used to construct a phylogenetic tree using the Maximum Likelihood (ML) approach (Kumar et al., 2016) with bootstrap analysis (heuristic search of 1000 replicates) by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances and all positions containing gaps and missing data were eliminated.

3. Results

The investigated tomato hind, *Cephalopholis sonnerati*, was naturally infected with a digenean parasite with a particular location for the intestine in 18 out of thirty (60%) fish specimens. This parasitic species was identified as *Bivesicula claviformis* Yamaguti, 1934. The parasite's mean intensity in each parasitized fish does not exceed six.

3.1. Description

The body was spindle-shaped, oculate, and covered with minute spines. There were no oral or ventral suckers. Pharynx had oval-shaped. Oesophagus was narrow. Ceca were simple and ended at the level of the posterior end of the testis. Location of a single large testis was middle and post-equatorial. Cirrus sac was oval and situated in the middle portion of the body. External vesicula seminalis was located immediately in front of the cirrus sac. Internal vesicula seminalis was found near the cirrus sac's anterior end. The space inside the cirrus sac was being filled by pars prostatica. Genital atrium was small and inconspicuous. Genital pore was equatorial. Ovary was sub-median, spherical, and on the right side of the body. Shell gland was posteromedial to the ovary. Uterus extends beyond the posterior end of the testis and then ascends. About 25 eggs were collapsed. Numerous vitelline follicles extending from the oesophageal level to the level of the posterior end of the testis make up vitellaria. The vitelline reservoir was dorsal, between ovary and testis. Excretory vesicle was V-shaped with arms that reached the level of the oesophagus. Excretory pore was located near the body's posterior extremity (Figs. 1 and 2).

3.2. Measurements

Body 1.043–1.546 (1.223) long × 0.651–0.692 (0.670) wide, pharynx 0.143–0.221 (0.168) long × 0.173–0.232 (0.183) wide, testis 0.185–0.256 (0.221) long × 0.183–0.264 (0.242) wide, cirrus sac 0.258–0.340 (0.291) long × 0.215–0.272 (0.252) wide, ovary 0.139–0.146 (0.142) long × 0.127–0.198 (0.183) wide, eggs 0.078–0.089 (0.085) long × 0.038–0.054 (0.047) wide (Table 1).

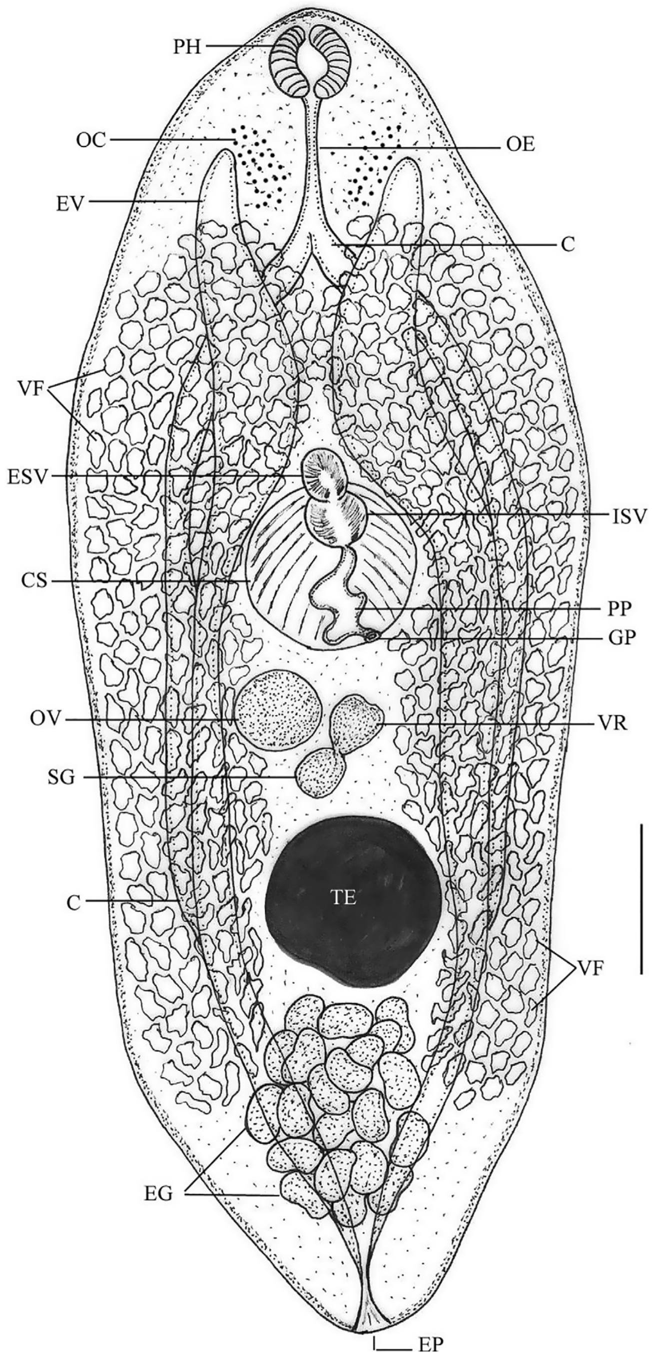


Fig. 2. Line drawing for the whole mount of the recovered *Bivesicula claviformis*. Scale bar = 200 μm. Note: C, ceca; CS, cirrus sac; EG, eggs; EP, excretory pore; ESV; external seminal vesicle; EV, excretory vesicle; GP, genital pore; ISV, internal seminal vesicle; OC, ocular region; OE, oesophagus; OV, ovary; PH, pharynx; PP, pars prostatica; SG, shell gland; SP, spinules; TE, testis; UT, uterus; VF, vitelline follicles; VR, vitelline reservoir.

3.3. Molecular analysis

3.3.1. 18S rRNA gene

The examined digenean species' partial *18S rRNA* sequence was 980 bp with a GC content of 51.4% and deposited in GenBank under

Table 1
Comparative measurements of *Bivesicula claviformis* collected in this study and other generic species reported previously.

Comparable parameters		Yamaguti (1934)	Nagaty (1948)	Manter (1961)	Fischthal and Kuntz (1965)	Cribb et al. (1994)	The present study (2022)
Host		*Seriola quinquoradiata, Parapristipoma trilineatum*	*Serranus fasciatus*	*Epinephelus merra*	*Epinephelus fasciatus*	*Epinephelus quoyanus*	*Cephalopholis sonnerati*
Locality		Inland Sea	Abu Luli, Red Sea	Fiji	North Borneo	Heron Island	Saudi Arabia
Infection site		Small intestine	Intestine	Intestine	Small intestine	Intestine	Intestine
Body	L	0.84–1.25	1.3–1.7	1.373–1.600	1.040–1.510	0.611–1.665 (1.205)	1.043–1.546 (1.223)
	W	0.36–0.55	0.519–0.572	0.603–0.710	0.645–0.680	0.203–0.443 (0.345)	0.651–0.692 (0.670)
Pharynx	L	0.095–0.13	0.167–0.180	–	0.140–0.201	0.078–0.158 (0.119)	0.143–0.221 (0.168)
	W	0.1–0.16	0.153–0.239	–	0.172–0.218	0.084–0.142 (0.125)	0.173–0.232 (0.183)
Testis	L	0.16–0.18	0.203	–	0.182–0.235	0.039–0.150 (0.107)	0.185–0.256 (0.221)
	W	0.13–0.21	0.261	–	0.181–0.290	0.036–0.159 (0.099)	0.183–0.264 (0.242)
Cirrus sac	L	0.15–0.25	–	–	0.242–0.336	0.094–0.303 (0.199)	0.258–0.340 (0.291)
	W	0.11–0.14	–	–	0.201–0.266	0.066–0.123 (0.100)	0.215–0.272 (0.252)
Ovary	L	0.074–0.11	0.135	–	0.136–0.142	0.050–0.100 (0.083)	0.139–0.146 (0.142)
	W	0.06–0.11	0.104	–	0.125–0.194	0.048–0.094 (0.078)	0.127–0.198 (0.183)
Eggs	L	0.087	0.081–0.09	0.082–0.096	0.076–0.082	0.075–0.083 (0.079)	0.078–0.089 (0.085)
	W	0.047	0.050–0.086	0.037–0.048	0.037–0.055	0.035–0.052 (0.042)	0.038–0.054 (0.047)

Table 2
Digenea species used in the analysis of *18S rRNA* sequence for *Bivesicula claviformis*.

Parasite species	Order, Family	Host species	% Identity	% GC content	Reference
AJ287485.1 Bivesicula claviformis	Azygiida, Bivesiculidae	*Epinephelus quoyanus*	99.39	50.9	Cribb et al. (2001)
AY222099.1 Bivesicula unexpecta	Azygiida, Bivesiculidae	*Acanthochromis polyacanthus*	96.73	51.3	Olson et al. (2003)
AY222100.1 Bivesicula fusiformis	Azygiida, Bivesiculidae	*Atherinomorus lacunosus*	94.30	49.9	Olson et al. (2003)
AJ287557.1 Paucivitellosus fragilis	Azygiida, Bivesiculidae	*Crenimugil crenilabis*	94.29	50.2	Cribb et al. (2001)
AY222110.1 Indosolenorchis hirudinaceus	Plagiorchiida, Paramphistomidae	*Dugong dugon*	91.50	51.1	Olson et al. (2003)
AJ287563.1 Preptetos caballeroi	Plagiorchiida, Lepocreadiidae	*Naso vlamingii*	91.34	49.9	Cribb et al. (2001)
MN700961.1 Skrjabinopsolus nudidorsalis	Plagiorchiida, Deropristidae	*Acipenser ruthenus*	91.18	50.4	Sokolov et al. (2020)
AY222135.1 Psilochasmus oxyurus	Plagiorchiida, Psilostomidae	*Anas platyrhynchos*	90.96	51.1	Olson et al. (2003)
AB551567.1 Liolope copulans	Strigeidida, Liolopidae	*Semisulcospira libertina*	90.92	51.9	Baba et al. (2011)
L06670.1 Tetracerasta blepta	Plagiorchiida, Lepocreadiidae	–	90.84	49.9	Blair and Barker (1993)
FJ550131.1 Paramphistomidae sp.	Plagiorchiida, Paramphistomidae	*Physa acuta*	90.81	51.8	Kraus et al. (2008)
AJ287497.1 Degeneria halosauri	Plagiorchiida, Gorgoderidae	*Halosauropsis macrochir*	90.60	50.9	Cribb et al. (2001)
AJ287481.1 Austroholorchis sprenti	Plagiorchiida, Lepocreadiidae	*Sillago ciliata*	90.54	49.7	Cribb et al. (2001)
AY222126.1 Gorgocephalus kyphosi	Plagiorchiida, Gorgocephalidae	*Kyphosus vaigiensis*	90.53	50.2	Olson et al. (2003)
L06567.1 Echinostoma caproni	Plagiorchiida, Echinostomatidae	–	90.37	50.6	Blair and Barker (1993)
DQ248207.1 Stephanostomum tantabiddii	Opisthorchiida, Acanthocolpidae	*Carangoides fulviguttatus*	90.37	50.6	Bray et al. (2005)
L06669.1 Gyliauchen sp.	Opisthorchiida, Gyliauchenidae	–	90.22	49.8	Blair and Barker (1993)
KY945918.1 Pegosomum saginatum	Plagiorchiida, Echinostomatidae	*Ardea alba*	90.16	52.4	Hirzmann et al. (2017)
AY829255.1 Spirorchid sp.	Strigeidida, Spirorchiidae	–	90.16	51.1	Brant et al. (2006)
KX506857.1 Diplodiscus mehrai	Plagiorchiida, Diplodiscidae	–	90.16	50.6	Besprozvannykh et al. (2016)
AY222119.1 Rhipidocotyle galeata	Strigeidida, Bucephalidae	*Eutrigla gurnardus*	90.07	49.8	Olson et al. (2003)
EU196357.1 Choanocotyle nematoides	Plagiorchiida, Choanocotylidae	*Emydura kreftii*	90.07	51.1	Tkach and Snyder (2007)
AY222123.1 Caecincola parvulus	Opisthorchiida, Cryptogonimidae	*Micropterus salmoides*	89.97	51.1	Olson et al. (2003)
FJ211228.1 Haploporus benedeni	Plagiorchiida, Haploporidae	*Liza ramado*	89.86	51.8	Blasco-Costa et al. (2008)
AY222129.1 Paraschistorchis zancli	Plagiorchiida, Apocreadiidae	*Zanclus cornutus*	89.84	50.8	Olson et al. (2003)
AY222122.1 Siphodera vinaledwardsii	Opisthorchiida, Cryptogonimidae	*Sciaenops ocellatus*	89.68	51	Olson et al. (2003)
MT280021.1 Hymenocotta mulli	Plagiorchiida, Haplosplanchnidae	–	89.57	50.3	Atopkin et al. (2020)
AY222083.1 Cotylaspis sp.	Aspidogastrea, Aspidogastridae	*Pelodiscus sinensis*	89.54	50.2	Olson et al. (2003)
AJ287523.1 Homalometron synagris	Plagiorchiida, Apocreadiidae	*Hemigymnus melapturus*	89.44	50.2	Cribb et al. (2001)
JQ627832.1 Philophthalmus gralli	Plagiorchiida, Philophthalmidae	*Tachuris rubrigastra*	89.43	51.8	Literak et al. (2013)
DQ482610.1 Multicalyx elegans	Aspidogastrea, Multicalycidae	*Callorhinchus milii*	89.27	50.5	Gao et al. (2006)

the accession number OK181883.1. The ML approach was used to align nucleotide sequence data from 32 taxa (including the out-group) over 944 positions to produce a phylogenetic dendrogram that represented two Digenea and Aspidogastrea subclasses (Table 2). The genus *Bivesicula* was validated by pairwise comparison with the GenBank *18S rRNA* gene data set, although non-species identification of previously deposited *Bivesicula* species was not (Fig. 3). The current phylogenetic dendrogram is divided into two main clades (Fig. 4), the first of which clustered taxa representing different orders of Digenea, and the second of which

```
                                    10         20         30         40         50         60         70         80         90
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK181883.1 B. claviformis   TGGTGCCAGC AGCCGCGGTA ACTCCAGCTC CAGAAGCGTA TATTAAAGTT GTTGCAGTTA AAAAGCTCGT AGTTGGATCT GGGTCGCATG
AJ287485.1 B. claviformis   .......... .......... .......... .......... .......... .......... .......... .......... ..........
AJ287557.1 P. fragilis      .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY222100.1 B. fusiformis    .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY222099.1 B. unexpecta     .......... .......... .......... .......... .......... .......... .......... .......... ..........

                                   100        110        120        130        140        150        160        170        180
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK181883.1 B. claviformis   GCTACATGTC GTGGCCTTTT TGTGAGTGGT TTGTTCTGCT CTCATTTGGG TCGATGTAGT GGCTGTGCAG CCTATCAGTC GTGTCCGTGA
AJ287485.1 B. claviformis   .......... .......... .......... .......... .......... .......... .......... ...T...... ..........
AJ287557.1 P. fragilis      ...T...A.. .......A.. ..C.G..... G.A.A..... .......... .......T.G ...C...... ...T...... .....T...T
AY222100.1 B. fusiformis    .T........ ...AT..G.. ....GA.... .AACA...T. T.....C... .......... A......... ...T...... .....T....
AY222099.1 B. unexpecta     ..CT...... ........CA .......... G.A.A..... .......... .......... ...C...... ...T...... .....T...T

                                   190        200        210        220        230        240        250        260        270
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK181883.1 B. claviformis   ATACGGGTGC CGGCTGGGTT GGTGGGCTAG TCTCATTGAC CTGTCGGCAT GCTTCCAGAT GCCTTTAAAC GGGTGTCGGG AGCGGACGAC
AJ287485.1 B. claviformis   .......... .......... .......... .......... .......... .......... .......... .......... ..........
AJ287557.1 P. fragilis      TA..A....T T....A...C ......T.-T ....GC...T .....A.... .......... .......... .......... ..........
AY222100.1 B. fusiformis    .A........ TT-TCAA... ........-T G.....CTT. T...TT.... ...C.AGA.. .......... .......... ..........
AY222099.1 B. unexpecta     TA........ T........C ......T.CT .......... .....A.... .......... ......C... .......... ..........

                                   280        290        300        310        320        330        340        350        360
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK181883.1 B. claviformis   ACGTTTACTT TGAACAAATT TGAGTGCTCA AAGGAGGCCT GTGTGCCTGA AAAGTCTTGC ATGGAATAAT AGAATAGGAC TTCGGTTCTA
AJ287485.1 B. claviformis   .......... .......... .......... ...C...... .......... .......... .......... .......... ..........
AJ287557.1 P. fragilis      .......... .......... .......... ...C...... ........A. .......... .......... G......... ..........
AY222100.1 B. fusiformis    .A........ .......... .......... ...C...... .......... ...T...... .......... .......... ..........
AY222099.1 B. unexpecta     .......... .......... .......... ...C...... .......... .......... .......... .......... ..........

                                   370        380        390        400        410        420        430        440        450
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK181883.1 B. claviformis   TTTTGTTGGT TTTCGGATCC GAAGTAATGG TTAAGAGGGA CAGACGGGGG CATTTGAATG GCGGTGTTAG AGGTGAAATT CTTGGATCGC
AJ287485.1 B. claviformis   .......... .......... .......... .......... .......... .......... .......... .......... ..........
AJ287557.1 P. fragilis      .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY222100.1 B. fusiformis    .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY222099.1 B. unexpecta     .......... .......... .......... .......... .......... .......... .......... .......... ..........

                                   460        470        480        490        500        510        520        530        540
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK181883.1 B. claviformis   CGCCAGTCAA GCTACAGCGA AAGCATTTGC CAAGAATGTT TTCATTGATC AGGAGCGAAA GTCAGAGCTT CGAAGACGAT CAGATACCGT
AJ287485.1 B. claviformis   .......... .......... .......... .......... .......... .......... .......G.. .......... ..........
AJ287557.1 P. fragilis      .......... .......... .......... .......... .......... .......... .......G.. .......... ..........
AY222100.1 B. fusiformis    .......... .......... .......... .......... .......... .......... .......G.. .......... ..........
AY222099.1 B. unexpecta     .......... .......... .......... .......... .......... .......... .......G.. .......... ..........

                                   550        560        570        580        590        600        610        620        630
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK181883.1 B. claviformis   CCTAGTTCTG ACCATAAACG ATGCCAACTG ACGATCCGTG GTGGTTCTGT TTTTGGCCCC GCGGGCAGTC TCCGGAAAAC CCTTAAGTCT
AJ287485.1 B. claviformis   .......... .......... .......... .......... .......... .......... .......... .......... ..........
AJ287557.1 P. fragilis      .......... .......... .......... .......... ........A. .......... .......... .......... ..........
AY222100.1 B. fusiformis    .......... .......... .......... .......... ....C...A. .....A.... .......... .......... ..........
AY222099.1 B. unexpecta     .......... .......... .......... .......... ........A. .......... .......... .......... ..........

                                   640        650        660        670        680        690        700        710        720
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK181883.1 B. claviformis   TTGGGCTCCG GGGGAAGTAT GGTTCCAAAG CTGAAACTTA AAGGAATTGA CGGAAGGGCA CCACCAGGAG TGGAGCCTGC GGCTTAATTC
AJ287485.1 B. claviformis   .......... .......... ....G..... .......... .......... .......... .......... .......... ..........
AJ287557.1 P. fragilis      .......... .......... ....G..... .......... .......... .......... .......... .......... ..........
AY222100.1 B. fusiformis    .......... .......... ....G..... .......... .......... .......... .......... .......... ..........
AY222099.1 B. unexpecta     .......... .......... ....G..... .......... .......... .......... .......... .......... ..........

                                   730        740        750        760        770        780        790        800        810
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK181883.1 B. claviformis   GACTCAACAC GGGAAAACAC ACCCGGCCCG GACACTGTGA GGATTGACAG ATTGATAGCT CTTTCTTGAT TCGGTGGTTG GTGGTGCATG
AJ287485.1 B. claviformis   .......... ........T. .......... .......... .......... .......... .......... .......... ..........
AJ287557.1 P. fragilis      .......... ........T. .......... .......... .......... .......... .......... .......... ..........
AY222100.1 B. fusiformis    .......... ........T. .......... .......... .......... .....G.... .......... .......... ..........
AY222099.1 B. unexpecta     .......... ........T. .......... .......... .......... .......... .......... .......... ..........

                                   820        830        840        850        860        870        880        890        900
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK181883.1 B. claviformis   GCCGTTCTTA GTTGGTGGAG CGATTTGTCT GGTTAATTCC GATAACGAAC GAGACTTTGG CCTGCTAAAT AGTATGCCTG TCCTCTGTGC
AJ287485.1 B. claviformis   .......... .......... .......... .......... .......... .......... .......... .......... ..........
AJ287557.1 P. fragilis      .......... .......... .......... .......... .......... ........CA .......... ....CAT... .T........
AY222100.1 B. fusiformis    .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY222099.1 B. unexpecta     .......... .......... .......... .......... .......... .......... .......... .......... ..........

                                   910        920        930        940        950        960        970        980
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK181883.1 B. claviformis   TCGTGCAGGG CGCGGCGTAC ACTGCCTCTT CGTGGGGTGG TGCTGTCGTT GTCCGGCGGG TGCGGCGGAG GTGTTTACTT
AJ287485.1 B. claviformis   .......... .......... .......... .......... .......... .......... .......C.. ..........
AJ287557.1 P. fragilis      .T..CT.... AC..T..... .......... .C........ ..T....... TC....T.A. .A.....C.. A.........
AY222100.1 B. fusiformis    ....C.T... TA...T..T. .......... .......... ..GC...... TC........ .A.....C.. ..........
AY222099.1 B. unexpecta     .......... T...T..... .......... .......... ..T....... .C......A. .A.....C.. ..........
```

Fig. 3. Sequence alignment of the partial *18S rRNA* gene of *Bivesicula claviformis* with the most closely related species (Only variable sites are shown. Dots represent bases identical to those of the first sequences, and dashes indicate gaps).

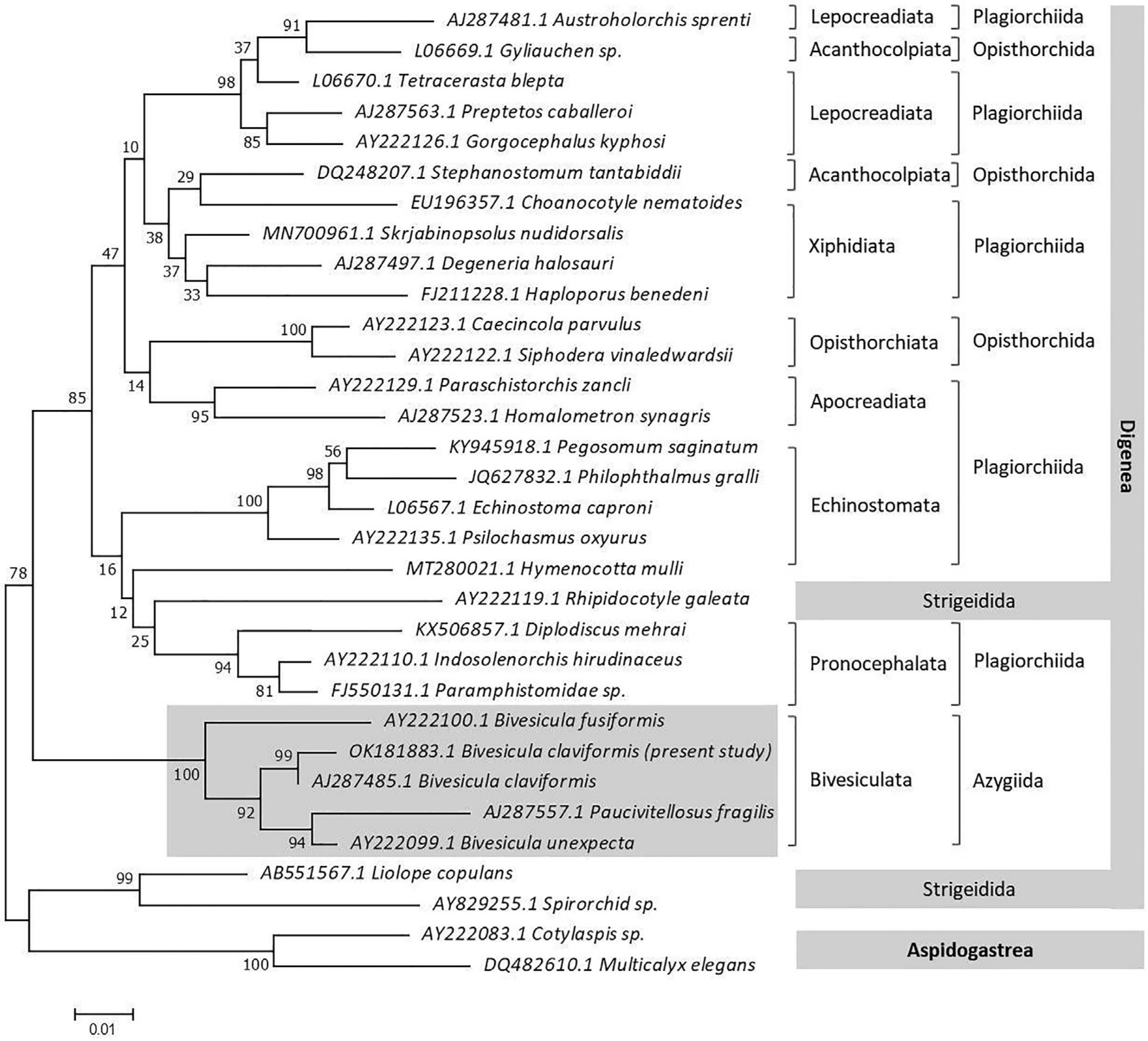


Fig. 4. Molecular Phylogenetic analysis by Maximum Likelihood method (ML) based on the Tamura-Nei model. The tree with the highest log likelihood (−6175.42) is shown.

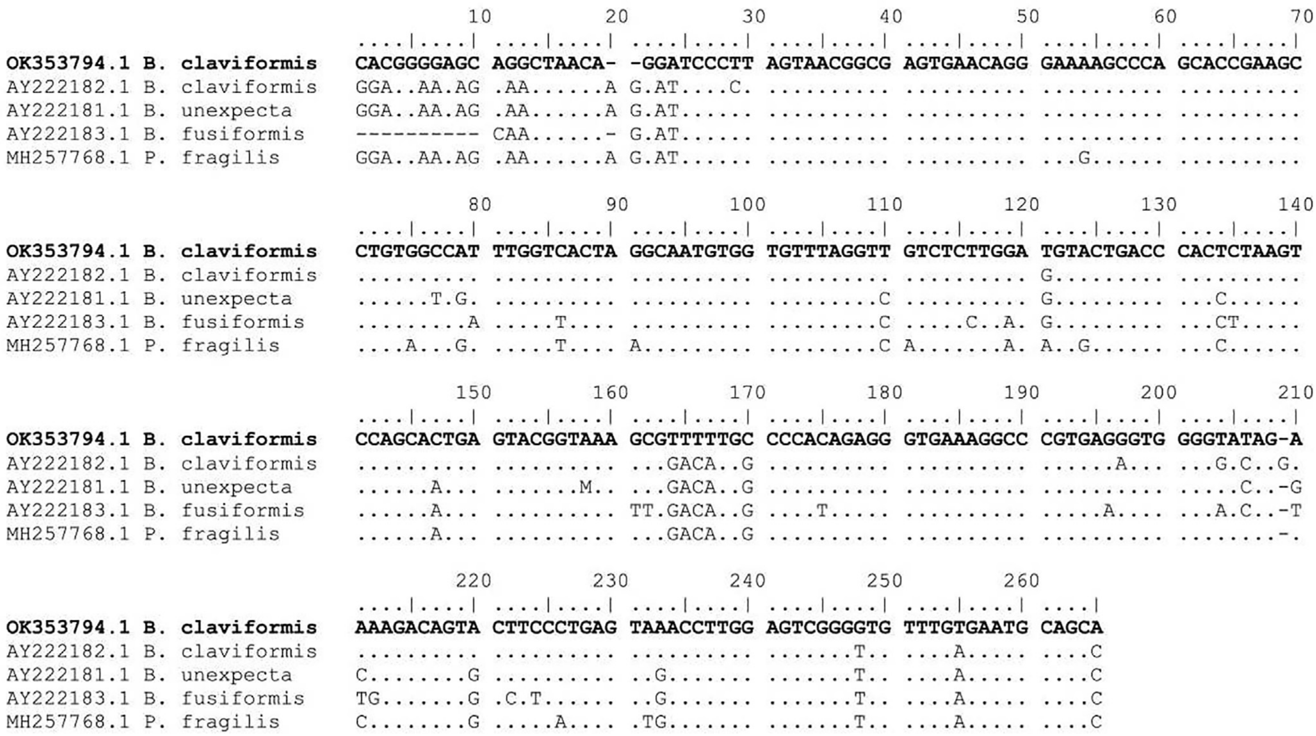
clustered two genera of Strigeidida (AB551567.1 *Liolope copulans* and AY829255.1 *Spirorchid* sp.), as well as two genera of Aspidogastrea (AY222083.1 *Cotylaspis* sp. and DQ482610.1 *Multicalyx elegans*), and the latter considered as out-group. The first major clade was split into two lineages, the first of which included Plagiorchiida and Opisthorchiida species with high support value (85). With a value of 100, the second lineage was highly supported by the Bivesiculidae family taxa. Cryptogonimidae appears as a sister group to Apocreadiidae. Haplosplanchnidae is also related to Bucephalidae + Diplodiscidae + Paramphistomidae. There was a strong relation (100) between Psilostomidae and Echinostomatidae + Philophthalmidae. There are sister relationships between Lepocreadiidae and Gyliauchenidae + Gorgocephalidae. A close relationship with strong support (98) was observed between Acanthocolpidae + Choanocotylidae and Deropristidae + Gorgoderidae + Haploporidae. Based on sequence comparisons, the sequence representing *B. claviformis* clustered with the sequence represented by AJ287485.1 for the previously deposited sequence for *B. claviformis*. These sequences resolved as a strongly supported monophyletic clade together with sequences representing *B. unexpecta* (AY222099.1), *B. fusiformis* (AY222100.1), and *Paucivitellosus hirudinaceus* (AJ287557.1).

3.3.2. 28S rRNA gene

The *28S rRNA* PCR result had a band size of 262 bp in length with 52.7% GC content, according to sequencing analysis. With the accession number OK353794.1, the sequence was submitted to the NCBI database. The query sequence was compared to other comparable digenean *28S rRNA* sequences in the GenBank database (Table 3, Fig. 5). The current phylogenetic tree was built using the ML method using 31 taxa (including the outgroup) and aligned over 226 positions (trimmed to match the shortest sequence length). The species examined formed two major clades (Fig. 6), the first of which includes 12 families from the subclass Digenea and five taxa from the Fellodistomidae family of Strigeidida. Whereas, the second clade contains taxa representing Cymnophalloidea and Monorchiidae (KJ648919.1 *Pseudobacciger cheneyae* and MN984478.1 *Lasiotocus trachinoti*, respectively) within Strigeidida and Plagiorchiida, in addition to the aspidogastrean outgroup taxa (AY222163.1 *Multicalyx elegans* and AY222165.1 *Cotylaspis* sp.). Fellodistomidae and Bivesiculidae are sibling families. The relationship between Troglotrematidae and Nanophyetidae has a well-supported clade (85). Opisthorchiidae + Liliatrematidae appears to be a sister group to Heterophyidae. This analysis demonstrated the basal position of Cymnophalloidea and

Table 3
Digenea species used in the analysis of *28S rRNA* sequence for *Bivesicula claviformis*.

Parasite species	Order, Family	Host species	% Identity	% GC content	Reference
AY222182.1 Bivesicula claviformis	Azygiida, Bivesiculidae	*Epinephelus quoyanus*	94.02	53.2	Olson et al. (2003)
AY222181.1 Bivesicula unexpecta	Azygiida, Bivesiculidae	*Acanthochromis polyacanthus*	91.60	53.3	Olson et al. (2003)
AY222183.1 Bivesicula fusiformis	Azygiida, Bivesiculidae	*Atherinomorus lacunosus*	87.95	52.7	Olson et al. (2003)
MH257768.1 Paucivitellosus fragilis	Azygiida, Bivesiculidae	*Clypeomorus batillariaeformis*	87.50	52.3	Huston et al. (2018)
KC489791.1 Acanthostomum burminis	Opisthorchiida, Cryptogonimidae	*Xenochrophis piscator*	86.22	53.4	Jayawardena et al. (2013)
AJ405298.1 Steringophorus thulini	Strigeidida, Fellodistomidae	*Coryphaenoides leptolepis*	85.60	50.8	Bray et al. (1999)
AB521800.1 Euryhelmis costaricensis	Opisthorchiida, Heterophyidae	*Martes melampus*	85.26	51.8	Sato et al. (2010)
FJ788492.1 Postlepidapedon uberis	Plagiorchiida, Lepocreadiidae	*Choerodon venustus*	85.26	52.9	Bray et al. (2009)
MW000405.1 Metagonimoides sp.	Opisthorchiida, Heterophyidae	*Juga* sp.	84.74	52.5	Preston et al. (2020)
AY222255.1 Zalophotrema hepaticum	Plagiorchiida, Brachycladiidae	*Zalophus californianus*	84.74	55.2	Olson et al. (2003)
KR703279.1 Brachycladium goliath	Plagiorchiida, Brachycladiidae	*Balaenoptera acutorostrata*	84.80	51.7	Briscoe et al. (2016)
AY222217.1 Taprobanella bicaudata	Plagiorchiida, Rhabdiopoeidae	*Dugong dugon*	84.80	53.2	Olson et al. (2003)
MG806918.1 Apophallus zalophi	Opisthorchiida, Heterophyidae	*Callorhinus ursinus*	85.42	53.8	Kuzmina et al. (2018)
KT865201.1 Proctoeces cf. *lintoni*	Strigeidida, Fellodistomidae	*Sicyases sanguineus*	84.86	49.2	Oliva et al. (2015)
MN984478.1 Lasiotocus trachinoti	Plagiorchiida, Monorchiidae	*Trachinotus carolinus*	84.77	51.1	Panyi et al. (2020)
MF099790.1 Opisthorchis felineus	Opisthorchiida, Opisthorchiidae	Cat	84.86	54	Dao et al. (2017)
MT303945.1 Liliatrema skrjabini	Plagiorchiida, Liliatrematidae	–	84.86	53.5	Sokolov et al. (2020)
AY222283.1 Olssonium turneri	Strigeidida, Fellodistomidae	*Alepocephalus agassizii*	84.86	52.9	Olson et al. (2003)
MK482053.1 Metorchis orientalis	Opisthorchiida, Opisthorchiidae	Duck	84.46	51.5	Qiu et al. (2019)
EF654661.1 Clonorchis sinensis	Opisthorchiida, Opisthorchiidae	–	84.46	52.9	Lee et al. (2007)
AY222231.1 Caecincola parvulus	Opisthorchiida, Cryptogonimidae	*Micropterus salmoides*	84.46	53.9	Olson et al. (2003)
KJ648919.1 Pseudobacciger cheneyae	Strigeidida, Cymnophalloidea	*Chromis weberi*	84.92	50.2	Sun et al. (2014)
HM172630.1 Paragonimus westermani	Plagiorchiida, Troglotrematidae	–	84.43	54.5	Devi et al. (2010)
KX354834.1 Campula oblonga	Plagiorchiida, Brachycladiidae	East Asian finless porpoise	84.52	55.6	Wan et al. (2016)
AY222282.1 Fellodistomum fellis	Strigeidida, Fellodistomidae	*Anarhichas lupus*	84.40	52.8	Olson et al. (2003)
KJ425462.1 Coomera brayi	Strigeidida, Fellodistomidae	*Monodactylus argenteus*	84	52.5	Cribb et al. (2014)
MZ345681.1 Bianium plicitum	Plagiorchiida, Lepocreadiidae	*Sphoeroides testudineus*	84	52.9	Curran (2021)
MW000381.1 Nanophyetus salmincola	Plagiorchiida, Nanophyetidae	*Juga* sp.	84	53.2	Preston et al. (2020)
AY222163.1 Multicalyx elegans	Aspidogastrea, Multicalycidae	*Callorhinchus milii*	79	49.5	Olson et al. (2003)
AY222165.1 Cotylaspis sp.	Aspidogastrea, Aspidogastridae	*Pelodiscus sinensis*	79	52.1	Olson et al. (2003)



```
                                    10         20         30         40         50         60         70
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK353794.1 B. claviformis   CACGGGGAGC AGGCTAACA- -GGATCCCTT AGTAACGGCG AGTGAACAGG GAAAAGCCCA GCACCGAAGC
AY222182.1 B. claviformis   GGA..AA.AG .AA......A G.AT....C. .......... .......... .......... ..........
AY222181.1 B. unexpecta     GGA..AA.AG .AA......A G.AT...... .......... .......... .......... ..........
AY222183.1 B. fusiformis    ---------- CAA......- G.AT...... .......... .......... .......... ..........
MH257768.1 P. fragilis      GGA..AA.AG .AA......A G.AT...... .......... .......... ...G...... ..........

                                    80         90        100        110        120        130        140
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK353794.1 B. claviformis   CTGTGGCCAT TTGGTCACTA GGCAATGTGG TGTTTAGGTT GTCTCTTGGA TGTACTGACC CACTCTAAGT
AY222182.1 B. claviformis   .......... .......... .......... .......... .......... G......... ..........
AY222181.1 B. unexpecta     ......T.G. .......... .......... .........C .......... G......... ...C......
AY222183.1 B. fusiformis    .........A .....T.... .......... .........C .....C..A. G......... ...CT.....
MH257768.1 P. fragilis      ....A...G. .....T.... A......... .........C A.......A. A..G...... ...C......

                                   150        160        170        180        190        200        210
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OK353794.1 B. claviformis   CCAGCACTGA GTACGGTAAA GCGTTTTTGC CCCACAGAGG GTGAAAGGCC CGTGAGGGTG GGGTATAG-A
AY222182.1 B. claviformis   .......... .......... ...GACA..G .......... .......... ......A... ...G.C..G.
AY222181.1 B. unexpecta     ......A... .......M.. ...GACA..G .......... .......... .......... .....C..-G
AY222183.1 B. fusiformis    ......A... .......... TT.GACA..G ....T..... .......... .....A.... ...A.C..-T
MH257768.1 P. fragilis      ......A... .......... ...GACA..G .......... .......... .......... ........-.

                                   220        230        240        250        260
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|
OK353794.1 B. claviformis   AAAGACAGTA CTTCCCTGAG TAAACCTTGG AGTCGGGGTG TTTGTGAATG CAGCA
AY222182.1 B. claviformis   .......... .......... .......... .......T.. ....A..... ....C
AY222181.1 B. unexpecta     C........G .......... ..G....... .......T.. ....A..... ....C
AY222183.1 B. fusiformis    TG.......G .C.T...... ..G....... .......T.. ....A..... ....C
MH257768.1 P. fragilis      C........G .....A.... .TG....... .......T.. ....A..... ....C
```

Fig. 5. Sequence alignment of the partial *28S rRNA* gene of *Bivesicula claviformis* with the most closely related species (Only variable sites are shown. Dots represent bases identical to those of the first sequences, and dashes indicate gaps).

Monorchiidae. There was a weak support (8) relationship between Cryptogonimidae and Lepocreadiidae. Among Bivesiculidae, the maximum identity (94.02%) with the lowest divergent value was recorded between the present digenean species and *Bivesicula claviformis* (AY222182.1) followed by *B. unexpecta* (91.60%, AY222181.1), *B. fusiformis* (87.95%, AY222183.1), and *Paucivitel-*

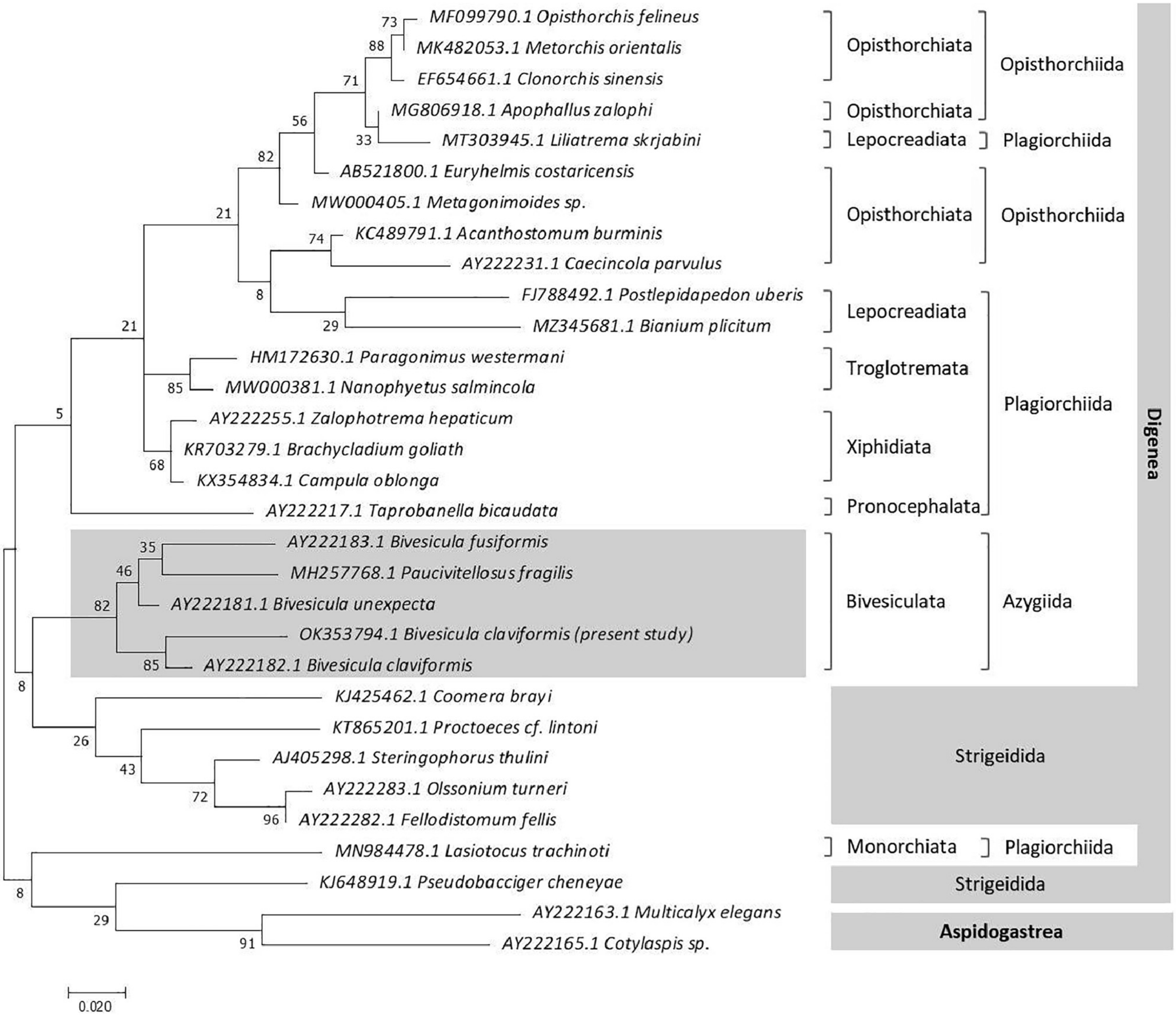


Fig. 6. Molecular Phylogenetic analysis by Maximum Likelihood method (ML) based on the Tamura-Nei model. The tree with the highest log likelihood (−1839.73) is shown.

losus hirudinaceus (87.50%, MH257768.1). The monophyly of *Bivesicula* species was corroborated by the sister group *Paucivitellosus* in general. The query sequence *B. claviformis* is well-matched and placed into the Azygiida with special reference to Bivesiculidae, with a close relationship to the previously known *B. claviformis* (AY222182.1) in a well-supported taxon (85).

4. Discussion

Although several studies on marine fish parasites from the Red Sea have been conducted (Al-Nabati et al., 2021), the Digenea constitutes the largest group of internal metazoan parasites (Cribb et al., 2001). Only 18 of the total 30 fish evaluated in this study exhibited a 60% infection rate for *B. claviformis* in the intestinal region. This agreed with Yamaguti (1971), Shen (1982), Gu and Shen (1983) and Cribb et al. (1994) documented *Bivesicula* digenea as intestinal parasites in marine fish.

At the morphological and morphometric level, the current bivesculid species is compatible with other *Bivesicula* species that have occupied many hosts in several geographical areas by possessing all the species' characteristics exceptions. The newly discovered *Bivesicula* species is quite similar to the previously described *Bivesicula claviformis* Yamaguti, 1934. The identification key was emphasized that the absence of the oral and ventral sucker, the presence of muscular pharynx, the intestine and vitellaria extended backward beyond the testis, the uterus entirely post-testicular, the arms of excretory vesicle extend well anterior to caeca, and the cirrus sac overlapped the midlevel of the body and its level could easily vary depending on the extent of the body contraction, as the main distinguishing features for the differentiation of *Bivesicula* species. The present study agreed with the previous data of Yamaguti (1934), Le Zotte (1954), Cable and Nahhas (1962), Fischthal and Kuntz (1965), Cribb et al. (1994), and Trieu et al. (2015) indicated that the oral sucker was the pharynx in all bivesiculids, and that the so-called pharynx was a muscular enlargement of the oesophagus. Our specimens fall within the range suggested by Fischthal and Kuntz (1965) for those infecting the intestine of *Epinephelus fasciatus* (Serranidae) from North Borneo in East Malaysia.

Bivesicula species have a wide range of hosts, but only seven families have been identified so far: Congridae, Megalopidae, Scombridae, Serranidae, Lutjanidae (Yamaguti, 1938, 1939; Nagaty, 1948; Manter, 1961; Fischthal and Kuntz, 1965; Machida et al., 1970; Gu and Shen, 1983; Shen, 1995; Lester and Sewell, 1990; Cribb et al., 1994; Shimazu and Machida, 1995; Rigby et al., 1997; Cribb et al., 1998; Nahhas et al., 2004), Holocentridae (Koryakovtseva, 1984), Synbranchidae (Shimazu, 2013), and Apogonidae (Trieu et al., 2015). Although the *Bivesicula* species was described from a carangid (type-host) and a lutjanid by Yamaguti (1934), the overwhelming majority of records are from serranids. Our findings represent the first evidence of endoparasitic *Bivesicula claviformis* infection in the serranid fish *Cephalopholis sonnerati*, which inhabits the Red Sea (Saudi Arabia).

In the last three decades, the inclusion of molecular research into taxonomy has become an essential tool for species determination (Al-Hoshani et al., 2021; Al-Quraishy et al., 2021). Although morphological descriptions of species are still extremely successful, combining morphological and genetic approaches improves species identification, particularly when complexes of species are present (Cribb et al., 2016; Blasco-Costa et al., 2016). The *18S*, *ITS1-5.8S-ITS2*, or *28S rDNA* regions are used in the majority of taxonomic and systematic investigations that employ molecular analysis for species descriptions. We were able to offer phylogenetic resolution and a good estimate of the recovered digenean species by integrating data from two nuclear ribosomal RNA (18S and 28S) genes. This agreed with Cribb et al. (2001), who utilized complete *ssrDNA* sequences from 75 digenean species representing 55 families, as well as 56 adult and larval morphological characters for these families, to determine the interrelationships of constituent groupings.

The current phylogeny is divided into two subclasses: Aspidogastrea and Digenea, with the latter being the most abundant with 30 families. Because Aspidobothrea taxa have a larger and more loculate ventral sucker, they were included as an outgroup. While, Digenea species have an oral and ventral sucker, the latter being a modification of the posterior adhesive organ or being eliminated. The Bivesiculata is represented by the Bivesiculidae family, which is from the digenean tree's basal lineage. It is agreed with Olson et al. (2003) that this position might be owing to the primitive absence of suckers being a derived state, or the development of suckers in the Diplostomida and the remainder of the Plagiorchiata occurring independently. In the present phylogeny, the Pronocephalata and Bivesiculata are sister taxa. This grouping is supported by the absence of the oral sucker or pharynx as described by Pearson (1992). Furthermore, there was a relationship with Diplodiscidae, which is compatible with Olson et al. (2003), who indicated that this family lacks the ventral sucker. According to Manter (1966), the Lepocreadiata includes two families: the Lepocreadiidae and the Gorgocephalidae, which are both characterized by unusual morphology, including an oral sucker with tentacles, a single caecum with a non-terminal ventral opening in the forebody, and a huge pocketed genital atrium opening dorsally. The current phylogeny revealed the Xiphidiata, which is regarded as the Digenea's crown clade and consists of three families: Brachycladiidae, Deropristidae, and Gorgoderidae; the latter two of which are sister taxa. The existence of a penetrating stylet in their cercariae, which represented the term Xiphidiata, supported the union of these families, according to Olson et al. (2003).

5. Conclusion

The validity and position of *Bivesicula claviformis* within the Bivesiculidae are supported by morphological and molecular phylogenetic evidence. This is the first time a genetic sequence for *Bivesicula* species has been discovered. Furthermore, Saudi Arabia represents a novel geographic distribution for *C. sonnerati*'s digenean parasite species. More samples and genetic markers should be used in future studies to have a better understating of this group of digenean 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.

Acknowledgements

Authors extend their appreciation to Zoology Department, College of Science, King Saud University (Saudi Arabia); for providing all facilities to complete this work.

References

Abdel-Gaber, R., Al Quraishy, S., Dkhil, M.A., Abu Hawsah, M., Bakr, L., Maher, S., 2020. Morphological and molecular analyses of *Paropecoelus saudiae* so. nov. (Plagiorchiida: Opecoelidae), a trematoda parasite of *Parupeneus rubescens* (Mullidae) from the Arabian Gulf. J. King Saud Univ. Sci. 32, 2243–2253.

Al-Hoshani, N., Al-Quraishy, S., Murshed, M., Aljawdah, H.M.A., Mares, M.M., Alghamdi, J., Al-Shaebi, E.M., Dkhil, M.A., Abdel-Gaber, R., 2021. *Cucullanus bulbosus* (Lane, 1916) Barreto, 1918 (Nematoda, Cucullanidae) from the common ponyfish *Leiognathus equulus* (Leiognathidae): Morphology and molecular study. Microb. Pathog. 154, 104821.

Al-Nabati, E., Al, S., Al-Quraishy, S., Alajmi, R., Al-Shaebi, E.M., Aljawdah, H.M.A., Dkhil, M.A., Abdel-Gaber, R., 2021. *Heteromicrocotyla polyorchis* Unnithan, 1961 (Monogenea: Heteromicrocotylidae), a gill parasite of the yellow-spotted trevally, *Carangoides fulvoguttatus* (Carangidae) from Saudi Arabia: Morphology and phylogeny. Microb. Pathog. 160, 105165.

Al-Quraishy, S., Dkhil, M.A., AL-Hoshani,, N.,, Alhafidh,, W.,, Abdel-Gaber,, R.,, 2021. First molecular data and morphological re-description of two copepod species, *Hatschekia sargi* and *Hatschekia leptoscari*, as parasites on *Parupeneus rubescens* in the Arabian Gulf. J. King Saud Univ. Sci. 33, 101290.

Atopkin, D.M., Besprozvannykh, V.V., Ha, N.D., Nguyen, H.V., Nguyen, T.V., 2020. New species of *Parasaccocoelium* (Haploporidae) and new genus *Pseudohaplosplanchnus* (Haplosplanchnidae) from mullet fish of Russian Far East and Vietnam: morphological and molecular data. https://www.ncbi.nlm.nih.gov/nuccore/MT280021.1.

Baba, T., Hosoi, M., Urabe, M., Shimazu, T., Tochimoto, T., Hasegawa, H., 2011. *Liolope copulans* (Trematoda: Digenea: Liolopidae) parasitic in *Andrias japonicus* (Amphibia: Caudata: Cryptobranchidae) in Japan: life cycle and systematic position inferred from morphological and molecular evidence. Parasitol. Int. 60, 181–192.

Besprozvannykh, V.V., Rozhkovan, K.V., Ermolenko, A.V., 2016. Morphology, life cycles, and phylogenetic position of *Diplodiscus mehrai* Pande, 1937 and *D. japonicus* (Yamaguti, 1936) (Digenea: Diplodiscus). https://www.ncbi.nlm.nih.gov/nuccore/KX506857.1.

Blair, D., Barker, S.C., 1993. Affinities of the Gyliauchenidae: utility of the 18S rRNA gene for phylogenetic inference in the Digenea (Platyhelminthes). Int. J. Parasitol. 23, 527–532.

Blasco-Costa, I., Cutmore, S.C., Miller, T.L., Nolan, M.J., 2016. Molecular approaches to trematode systematics: 'Best practice' and implications for future study. Syst. Parasitol. 93, 295–306.

Blasco-Costa, I., Mantero, F.E., Gibson, D.I., Balbuena, J.A., Rega, J.A., Shvetsova, L.S., Kostadinova, A., 2008. A revision of the species of Saturnius Manter, 1969 (Digenea, Hemiuridae), parasites of mullets (Teleostei: Mugilidae). Syst. Parasitol. 71, 53–74.

Brant, S.V., Morgan, J.A., Mkoji, G.M., Snyder, S.D., Rajapakse, R.P., Loker, E.S., 2006. An approach to revealing blood fluke life cycles, taxonomy, and diversity: provision of key reference data including DNA sequence from single life cycle stages. J. Parasitol. 92 (1), 77–88.

Bray, R.A., Gibson, D.I., Zhang, J.Y., 1999. Urotrematidae (Platyhelminthes: Digenea) in Chinese freshwater fishes. Syst. Parasitol. 44, 193–200.

Bray, R.A., Waeschenbach, A., Cribb, T.H., Weedall, G.D., Dyal, P., Littlewood, D.T.J., 2009. The phylogeny of the Lepocreadioidea (Platyhelminthes: Digenea) inferred from nuclear and mitochondrial genes: Implications for their systematics and evolution. Acta Parasitol. 54, 310–329.

Bray, R.A., Webster, B.L., Bartoli, P., Littlewood, D.T.J., 2005. A molecular phylogenetic study of the Acanthocolpidae (Digenea). Acta Parasitol. 50, 281–291.

Briscoe, A.G., Bray, R.A., Brabec, J., Littlewood, D.T., 2016. The mitochondrial genome and ribosomal operon of *Brachycladium goliath* (Digenea: Brachycladiidae) recovered from a stranded minke whale. Parasitol. Int. 65 (3), 271–275.

Bush, A.O., Lafferty, K.D., Lotz, J.M., Shostak, A.W., 1997. Parasitology meets ecology on its own terms: Margolis et al. revisited. J. Parasitol. 83, 575–583.

Cable, R.M., Nahhas, F.M., 1962. *Bivesicula caribbensis* sp. n. (Trematoda: Digenea) and its life history. J. Parasitol. 48, 536–538.

Coil, W.H., Reid, W.A., Kuntz, R.E., 1965. Paucivitellosus fragilis gen. et sp. nov. (Bivesiculidae: Digenea), a parasite of Chelon troscheli from Formosa. Trans. Am. Microsc. Soc. 84 (3), 365–368.

Cribb, T.H., 2002. Superfamily Bivesiculoidea Yamaguti, 1934. In Gibson, D. I., A. Jones and R. A. Bray (eds.): Keys to the Trematoda, 1, pp. 25–29. CAB International and The Natural History Museum, Wallingford.

Cribb, T.H., Anderson, G.R., Adlard, R.D., Bray, R.A., 1998. A DNA-based demonstration of a three-host life-cycle for the Bivesiculidae (Platyhelminthes: Digenea). Int. J. Parasitol. 28, 1791–1795.

Cribb, T.H., Bott, N.J., Bray, R.A., McNamara, M.K.A., Miller, T.L., Nolan, M.J., Cutmore, S.C., 2014. Trematodes of the Great Barrier Reef, Australia: emerging patterns of diversity and richness in coral reef fishes. Int. J. Parasitol. 44, 929–939.

Cribb, T.H., Bray, R.A., 2010. Gut wash, body soak, blender, and heat-fixation: approaches to the effective collection, fixation and preservation of trematodes of fishes. Syst. Parasitol. 76, 1–7.

Cribb, T.H., Bray, R.A., Barker, S.C., 1994. Bivesiculidae and Haplosplanchnidae (Digenea) from fishes of the southern Great Barrier Reef, Australia. Syst. Parasitol. 28, 81–97.

Cribb, T.H., Bray, R.A., Diaz, P.E., Huston, D.C., Kudlai, O., Martin, S.B., Yong, R.Q.Y., Cutmore, S.C., 2016. Trematodes of fishes of the Indo-west Pacific: Told and untold richness. Syst. Parasitol. 93, 237–247.

Cribb, T.H., Bray, R.A., Littlewood, D.T.J., Pichelin, S.P., Herniou, E.A., 2001. The Digenea. In: Littlewood, D.T.J., Bray, R.A. (Eds.), Interrelationships of the Platyhelminthes. Taylor and Francis, London, pp. 168–185.

Curran, S.S., 2021. *Opechona chloroscombri* and *Opechona corkumi* n. sp. (Digenea: Lepocreadiidae) from the northern Gulf of Mexico with phylogenetic analysis based on 28S rDNA. J. Parasitol. 107 (4), 606–620.

Dao, T.H.T., Nguyen, T.G.T., Gabriel, S., Bui, K.L., Dorny,P., Blair, D., Le, T.H., 2017. The recently discovered *Opisthorchis* sp. in ducks in Vietnam: Insights of the genomic analysis for phylotaxonomic position in the family Opisthorchiidae (Opisthorchioidea: Trematoda: Platyhelminthes). https://www.ncbi.nlm.nih.gov/nuccore/MF099790.1.

Devi, K.R., Narain, K., Agatsuma, T., Blair, D., Nagataki, M., Wickramasinghe, S., Yatawara, L., Mahanta, J., 2010. Morphological and molecular characterization of *Paragonimus westermani* in northeastern India. Acta Trop. 116 (1), 31–38.

Fischthal, J.H., Kuntz, R.K., 1965. Digenetic trematodes of fishes from North Borneo (Malaysia). PHSWA 32 (1), 63–71.

Gao, Q., Chen, M., Nie, P., 2006. Phylogeny of the Subclass Aspidogastrea based on 18S rDNA Sequences. https://www.ncbi.nlm.nih.gov/nuccore/DQ482610.1.

Gu, C.D., Shen, J.W., 1983. Digenetic trematodes of fishes from the Xisha Islands, Guangdong Province, China. I. Stud. Mar. Sinica 20, 157–184.

Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hariri, K.I., Nichols, P., Krupp, F., Mishrigi, S., Barrania, A., Ali, F.A., Kedidi, S.M., 2000. Status of the living marine resources in the Red Sea and Gulf of Aden region and their management. Regional Organization for the Conservation of the Environment of the Red Sea and Gulf of Aden (PERSGA), Jeddah, Saudi Arabia. Report number: PERSGA Technical Series No. 4, Affiliation: PERSGA/The World Bank.

Hirzmann, J., Kley-Sonntag, A., Grossmann, E., Bauer, C., 2017. Molecular characterization of *Pegosomum* species (Trematoda, Echinostomatidae) from the gall bladder of a severe infected great egret in Germany. https://www.ncbi.nlm.nih.gov/nuccore/KY945918.1.

Huston, D.C., Cutmore, S.C., Cribb, T.H., 2018. Molecular systematics of the digenean community parasitizing the certhiid gastropod *Clypeomorus batillariaeformis* Habe & Kusage on the Great Barrier Reef. Parasitol. Int. 67, 722–735.

Indaryanto, F.R., Abdullah, M.F., Wardiatno, Y., Tiuria, R., Imai, H., 2015. A Description of *Lecithocladium angustiovum* (Digenea: Hemiuridae) in Short Mackerel, *Rastrelliger brachysoma* (Scombridae), of Indonesia. Trop. Life Sci. Res. 26 (1), 31–40.

Jayawardena, U.A., Tkach, V.V., Navaratne, A.N., Amerasinghe, P.H., Rajakaruna, R.S., 2013. Malformations and mortality in the Asian Common Toad induced by exposure to pleurolophocercous cercariae (Trematoda: Cryptogonimidae). Parasitol. Int. 62 (3), 246–252.

Justine, J.L., Beveridge, I., Boxshall, G.A., Bray, R.A., Moravec, F., Trilles, J.P., Whittington, I.D., 2010. An annotated list of parasites (Isopoda, Copepoda, Monogenea, Digenea, Cestoda and Nematoda) collected in groupers (Serranidae, Epinephelinae) in New Caledonia emphasizes parasite biodiversity in coral reef fish. Folia Parasitol. 57, 237–262.

Koryakovtseva, L.P., 1984. Morphological characteristics of trematodes from the family Bivesiculidae - parasites of marine fishes. Materialy Nauchnykh Konferentsii Vsesoyuznogo Obshchestva Germintologov 34, 29-34.

Kraus, T.J., Brant, S.V., Adema, C.M., 2008. Trematode cercariae from physid snails from the Middle Rio Grande. https://www.ncbi.nlm.nih.gov/nuccore/FJ550131.1.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Kuzmina, T.A., Tkach, V.V., Spraker, T.R., Lyons, E.T., Kudlai, O., 2018. Digeneans of northern fur seals *Callorhinus ursinus* (Pinnipedia: Otariidae) from five subpopulations on St. Paul Island, Alaska. Parasitol. Res. 117, 1079–1086.

Le Zotte, L.A., 1954. Studies on marine digenetic trematodes of Puerto Rico: The family Bivesiculidae, its biology and affinities. J. Parasit. 40, 148–162. 4 pls.

Lee, S.U., Chun, H.C., Huh, S., 2007. Molecular phylogeny of parasitic Platyhelminthes based on sequences of partial 28S rDNA D1 and mitochondrial cytochrome c oxidase subunit I. Korean J. Parasitol. 45 (3), 181–189.

Lester, R.J.G., Sewell, K.B., 1990. Checklist of parasites from Heron Island, Great Barrier Reef. Aust. J. Zool. 37, 101–128.

Literak, I., Heneberg, P., Sitko, J., Wetzel, E.J., Cardenas Callirgos, J.M., Capek, M., Valle Basto, D., Papousek, I., 2013. Eye trematode infection in small passerines in Peru caused by *Philophthalmus lucipetus*, an agent with a zoonotic potential spread by an invasive freshwater snail. Parasitol. Int. 62 (4), 390–396.

Machida, M., Ichihara, A., Kamegai, S., 1970. Digenetic trematodes collected from the fishes in the sea north of the Tsushima Islands. Mem. Natn. Sci. Mus. Tokyo 3, 101–112.

Mani, G.G., 1989. Morphology and life cycle of *Paucivitellosus hanumanthai* n. sp. (Trematoda: Bivesiculidae). Trans. Am. Microsc. Soc. 108, 21–26.

Manter, H.W., 1961. Studies on digenetic trematodes of fishes of Fiji. I. Families Haplosplanchnidae, Bivesiculidae, and Hemiuridae. PHSWA 28 (1), 67–74.

Manter, H.W., 1966. A peculiar trematode, *Gorgocephalus kyphosi* gen. et sp. n. (Lepocreadiidae: Gorgocephalinae subfam. n.), from a marine fish of South Australia. J. Parasitol. 52, 347–350.

Nagaty, H.F., 1948. Trematodes of fishes from the Red Sea. Part 4. On some new and known forms with a single testis. J. Parasit. 34, 355–363.

Nahhas, F.M., Nasser, H., Tam, J., 2004. Digenetic trematodes of marine fishes from Suva, Fiji: families: Acanthocolpidae, Lepocreadiidae, Bivesiculidae, Zoogonidae, Monorchiidae and description of a new species. Riv. Parassitol. 21, 33–48.

Oliva, M.E., Valdivia, I.M., Cardenas, L., Munoz, G., Geroge-Nascimento, M., 2015. A new species of Proctoeces and reinstatement of *Proctoeces humboldti* George-Nascimento and Quiroga 1983 (Digenea: Fellodistomidae) based on molecular and morphological evidence. https://www.ncbi.nlm.nih.gov/nuccore/KT865201.1.

Olson, P.D., Cribb, T.H., Tkach, V.V., Bray, R.A., Littlewood, D.T.J., 2003. Phylogeny and classification of the Digenea (Platyhelminthes : Trematoda). Int. J. Parasitol. 33, 733–755.

Panyi, A.J., Curran, S.S., Overstreet, R.M., 2020. Phylogenetic affinity of Genolopa (Digenea: Monorchiidae) with descriptions of two new species. Diversity 12 (2), 51.

Pearson, J.C., 1968. Observations on the morphology and life-cycle of *Paucivitellosus fragilis* Coil, Reid & Kuntz, 1965 (Trematoda: Bivesiculidae). Parasitology 58, 769–788.

Pearson, J.C., 1992. On the position of the digenean family Heronimidae an inquiry into a cladistic classification of the Digenea. Syst. Parasitol. 21, 81–166.

Preston, D.L., Layden, T.J., Segui, L.M., Falke, L.P., Brant, S.V., Novak, M., 2020. Trematode parasites exceed aquatic insect biomass in Oregon stream food webs. J. Anim. Ecol. 90 (3), 766–775.

Qiu, Y.Y., Su, X., Wang, C.R., 2019. Comparative analysis of Ribosomal DNA of *Clonorchis sinensis* and *Metorchis orientalis* about of human and animal health significance. https://www.ncbi.nlm.nih.gov/nuccore/MK482053.1.

Rigby, M.C., Holmes, J.C., Cribb, T.H., Morand, S., 1997. Patterns of species diversity in the gastrointestinal helminths of a coral reef fish, *Epinephelus merra* (Serranidae), from French Polynesia and the South Pacific Ocean. Can. J. Zool. 75, 1818–1827.

Rohde, K., Heap, M., 1998. Latitudinal differences in species and community richness and in community structure of metazoan endo- and ectoparasites of marine teleost fish. Int. J. Parasitol. 28, 461–474.

Sato, H., Ihara, S., Inaba, O., Une, Y., 2010. Identification of *Euryhelmis costaricensis* metacercariae in the skin of Tohoku hynobiid salamanders (*Hynobius lichenatus*), northeastern Honshu, Japan. J. Wildl. Dis. 46 (3), 832–842.

Shellem, C.T., Ellis, J.I., Coker, D.J., Berumen, M.L., 2021. Red Sea fish market assessments indicate high species diversity and potential overexploitation. Fish. Res. 239, 105922.

Shen, J.W., 1982. Three new species of genus *Bivesicula* Yamaguti, 1934 (Trematode [sic]: Bivesiculidae) from marine fishes of China. Oceanol. Limnol. Sin. 13, 570–576.

Shen, J.W., 1995. Notes on a new genus and species of Treptodemidae (Trematoda: Digenea). Stud. Mar. Sin. 36, 233–236.

Shimazu, T., 2013. Digeneans parasitic in freshwater fishes (Osteichthyes) of Japan. I. Aporocotylidae, Bivesiculidae and Haploporidae. Bull. Natl. Mus. Nat. Sci., Ser. A 39 (4), 167-184.

Shimazu, T., Machida, M., 1995. Some species of the genus *Bivesicula* (Digenea: Bivesiculidae), including three new species, from marine fishes of Japan and Palau. Bull. Natl. Sci. Mus., Tokyo, Ser. A, Zool. 21, 127–141.

Sokolov, S.G., Gordeev, I.I., Atopkin, D.M., 2020. Phylogenetic affiliation of the lepocreadiid trematodes parasitizing some marine fishes in the North-western Pacific. Mar. Biol. Res. 16 (5), 1–10.

Sun, D., Bray, R.A., Yong, R.Q., Cutmore, S.C., Cribb, T.H., 2014. *Pseudobacciger cheneyae* n. sp. (Digenea: Gymnophalloidea) from Weber's chromis (*Chromis weberi* Fowler & Bean) (Perciformes: Pomacentridae) at Lizard Island, Great Barrier Reef, Australia. Syst. Parasitol. 88 (2), 141-152.

Swainson, W., 1839. On the Natural History and Classification of Fishes, Amphibians, and Reptiles, or Monocardian Animals Vol. 2, 448.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22 (22), 4673–4680.

Tkach, V.V., Snyder, S.D., 2007. *Aptorchis megacetabulus* n. sp. (Platyhelminthes: Digenea) from the northern long-necked turtle, *Chelodina rugosa* (Pleurodira: Chelidae), in Australia. J. Parasitol. 93 (2), 404–408.

Trieu, N., Cutmore, S.C., Miller, T.L., Cribb, T.H., 2015. A species pair of *Bivesicula* Yamaguti, 1934 (Trematoda: Bivesiculidae) in unrelated Great Barrier Reef fishes: implications for the basis of speciation in coral reef fish trematodes. Syst. Parasitol. 91, 231–239.

Wan, X.L., Zheng, J.S., Li, W.X., Zeng, X.Y., Yang, J.W., Hao, Y.J., Wang, D., 2016. Survey on parasitic infections in the East Asian finless porpoise (*Neophocaena asiaeorientalis sunameri*) living off the Chinese Yellow/Bohai Sea coast. https://www.ncbi.nlm.nih.gov/nuccore/KX354834.1.

Wei, N.W.V., Wallace, C.C., Dai, C., Pillay, K.R.M., Chen, C.A., 2006. Analyses of the ribosomal internal Transcribed spacers (ITS) and the 5.8S gene indicate that extremely high rDNA heterogeneity is a unique feature in the scleractinian coral genus *Acropora* (Scleractinia; Acroporidae). Zool. Stud. 45 (3), 404–418.

WoRMS, 2021. Bivesiculidae Yamaguti, 1934. Accessed at: https://www.marinespecies.org/aphia.php?p=taxdetails&id=411241 on 2021-10-22.

Yamaguti, S., 1934. Studies on the helminth fauna of Japan. Part 2. Trematodes of fishes. I. Jpn. J. Zool. 5, 249–541.

Yamaguti, S., 1938. Studies on the Helminth Fauna of Japan. Part 21. Trematodes of Fishes, IV. Kyöto: Yamaguti, S, 139 pp.

Yamaguti, S., 1939. Studies on the helminth fauna of Japan. Part 26. Trematodes of fishes. VI. Jpn. J. Zool. 8 (2), 211–230.

Yamaguti, S., 1971. Synopsis of Digenetic Trematodes of Vertebrates. Keigaku Publishing Co, Tokyo.