



Original article

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

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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.

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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, *Paucicivitellosus* 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-

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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).

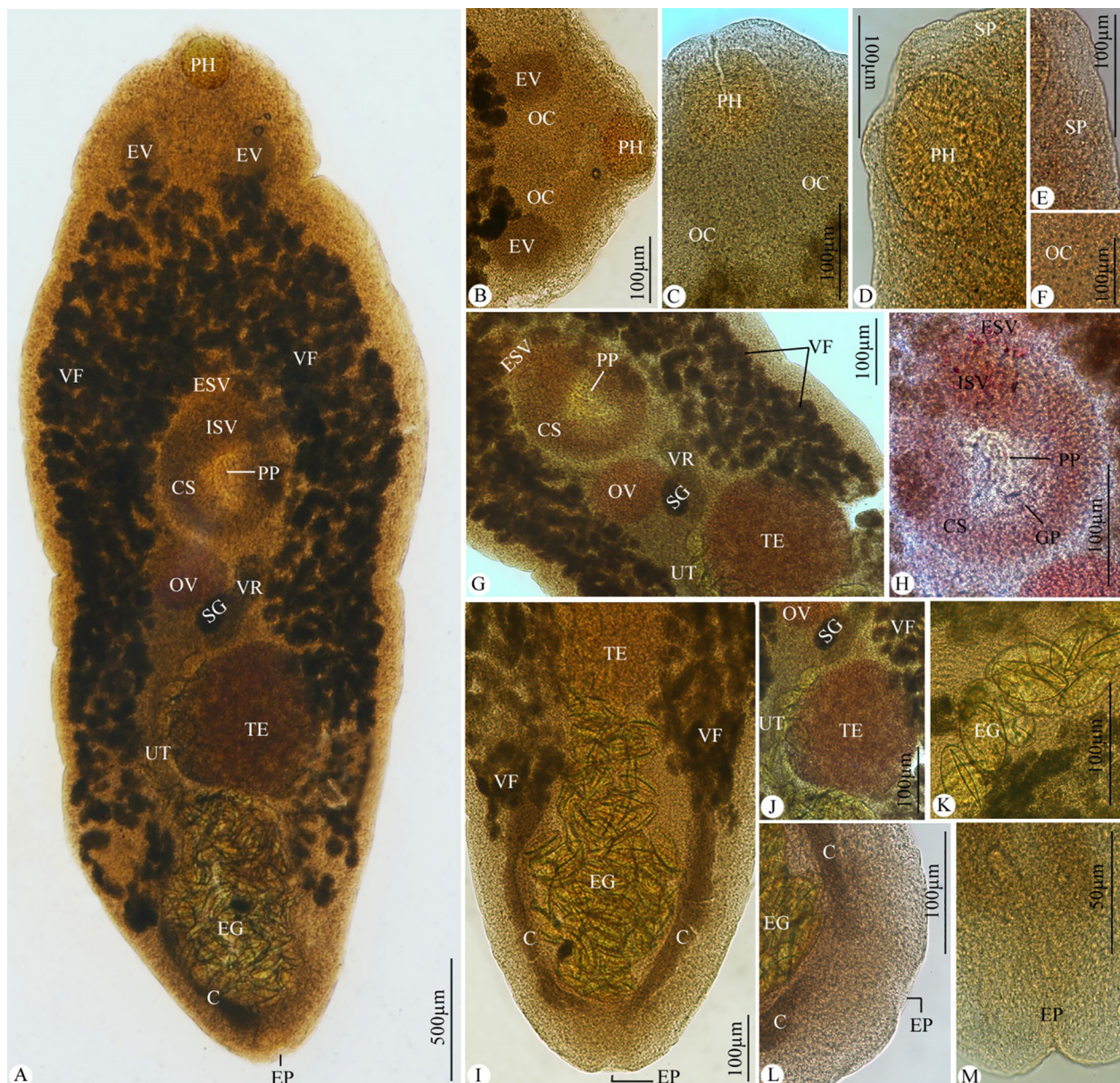


Fig. 1. Photomicrographs of *Bivesicula claviformis* infecting *Cephalopholis sonnerati*. (A) Whole-mount preparation. (B–F) Anterior portion of the body. (G, H) Middle portion of the body. (I–M) Posterior portion of the body. Note: C, ceca; CS, cirrus sac; EG, eggs; EP, excretory pore; ESV, external 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, tes-

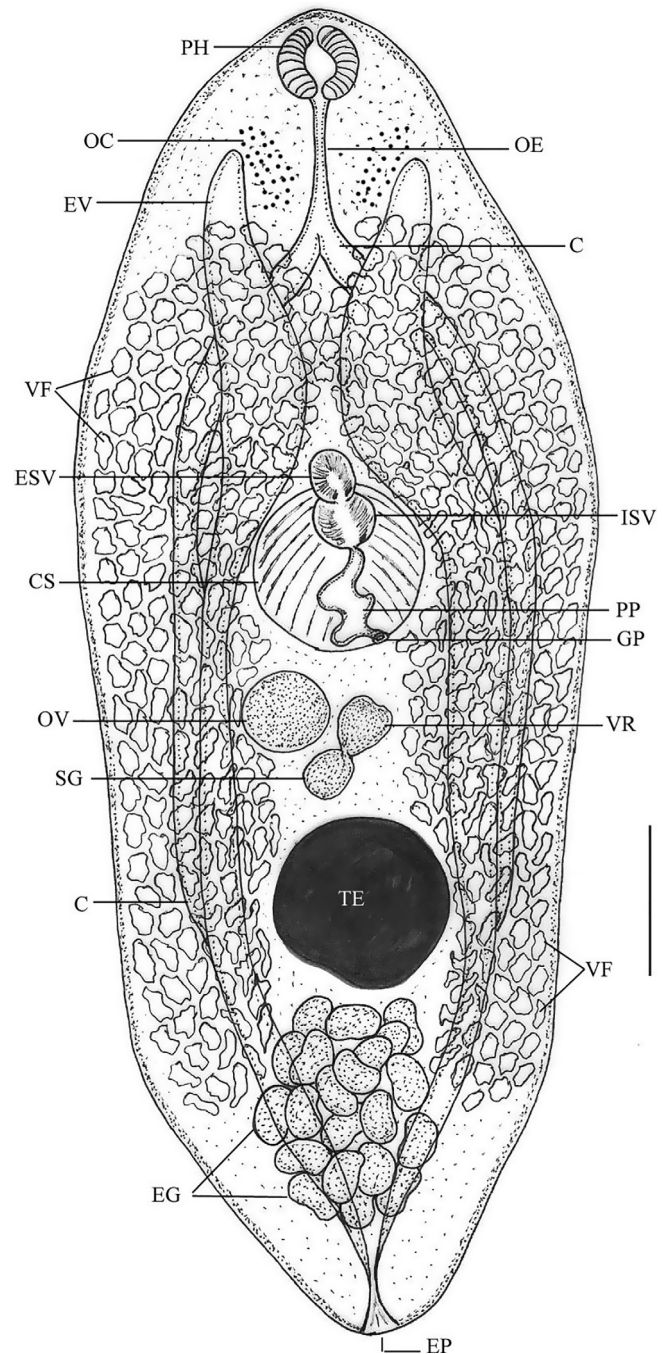


Fig. 2. Line drawing for the whole mount of the recovered *Bivesicula claviformis*. Scale bar = 200 µm. Note: C, ceca; CS, cirrus sac; EG, eggs; EP, excretory pore; ES, external seminal vesicle; EV, excretory vesicle; GP, genital pore; ISV, internal seminal vesicle; OC, ocular region; OE, oesophagus;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

tis 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).

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 1Comparative 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	<i>Seriola quinqueradiata</i> , <i>Parapristipoma trilineatum</i>	<i>Serranus fasciatus</i>	<i>Epinephelus merra</i>	<i>Epinephelus fasciatus</i>	<i>Epinephelus quoyanus</i>	<i>Cephalopholis sonnerati</i>
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 2Digenea species used in the analysis of 18S rRNA sequence for *Bivesicula claviformis*.

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

ison 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									
AJ287485.1	B. claviformis	TGGTGCCAGC	AGCCGCGGTA	ACTCCAGCTC	CAGAAGCGTA	TATTAAAGTT	GTTGCAGTTA	AAAAGCTCGT	AGTTGGATCT	GGGTCGCATG
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									
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..T	
AY222099.1	B. unexpecta	..CT.....	CA	G.A.A...	T...	C...	A...	C...	T...	T..T
		190	200	210	220	230	240	250	260	270
OK181883.1	B. claviformis	ATACGGGTGC	CGGCTGGGTT	GGTGGGCTAG	TCCTATTGAC	CTGTCCGCAT	GCTTCCAGAT	GCCTTTAAAC	GGGTGTGCGG	AGCGGACGAC
AJ287485.1	B. claviformis									
AJ287557.1	P. fragilis	TA..A...T	T...A...C	T..T	GC...T	A...	C...	T...	T..T	
AY222100.1	B. fusiformis	.A.....	TT-TCAA	-T	G....CTT	T...TT...	C.AGA	T...	T..T	
AY222099.1	B. unexpecta	TA.....	T.....C	T.CT	A...	C...	T...	T..T	T..T	
		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									
AJ287557.1	P. fragilis									
AY222100.1	B. fusiformis	.A.....								
AY222099.1	B. unexpecta									
		370	380	390	400	410	420	430	440	450
OK181883.1	B. claviformis	TTTTGTTGGT	TTTCGGATCC	GAAGTAATGG	TTAAGAGGGA	CAGACGGGGG	CATTGGAATG	CGCGTGTTAG	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	CAAGATGTTT	TTGATTGATC	AGGAGCGAAA	GTCAGAGCCT	CGAAGACGAT	CAGATACCGT
AJ287485.1	B. claviformis									
AJ287557.1	P. fragilis									
AY222100.1	B. fusiformis									
AY222099.1	B. unexpecta									
		550	560	570	580	590	600	610	620	630
OK181883.1	B. claviformis	CCTAGTTCTG	ACCATAAACG	ATGCCAATCG	ACGATCCGTG	GTGGTTCTGT	TTTTGGCCCC	CGCGGCAGTC	TCCGGAAAAC	CCTTAAGTCT
AJ287485.1	B. claviformis									
AJ287557.1	P. fragilis									
AY222100.1	B. fusiformis									
AY222099.1	B. unexpecta									
		640	650	660	670	680	690	700	710	720
OK181883.1	B. claviformis	TTGGGCTCCG	GGGGAAGTAT	GGTTCCAAAG	CTGAACTTA	AAGGAATTGA	CGGAAGGGCA	CCACCAGGAG	TGGAGCCTGC	GGCTTAATTC
AJ287485.1	B. claviformis									
AJ287557.1	P. fragilis									
AY222100.1	B. fusiformis									
AY222099.1	B. unexpecta									
		730	740	750	760	770	780	790	800	810
OK181883.1	B. claviformis	GACTCAACAC	GGGAAAACAC	ACCGGCCCCG	GACACTGTGA	GGATTGACAG	ATTGATAGCT	CTTCTTGAT	TCGGTGTTTG	GTGGTGCATG
AJ287485.1	B. claviformis									
AJ287557.1	P. fragilis									
AY222100.1	B. fusiformis									
AY222099.1	B. unexpecta									
		820	830	840	850	860	870	880	890	900
OK181883.1	B. claviformis	GCCGTTCTTA	GTTGGTGAG	CGATTGTTCT	GGTTAATTC	GATAACGAAC	GAGACTTTGG	CCTGCTAAAT	AGTATGCTG	TCCTCTGTGC
AJ287485.1	B. claviformis									
AJ287557.1	P. fragilis									
AY222100.1	B. fusiformis									
AY222099.1	B. unexpecta									
		910	920	930	940	950	960	970	980	
OK181883.1	B. claviformis	TCGTGCAGGG	CGCGCGGTAC	ACTGCCTCTT	CGTGGGGTGG	TGCTGTCGTT	GTCCGGCGGG	TGCGGCGGAG	GTGTTTACTT	
AJ287485.1	B. claviformis									
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...	TC...A...	A...	C...	A...	
AY222099.1	B. unexpecta	T..T...	T...	T...	C...	A...	A...	C...	A...	

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).

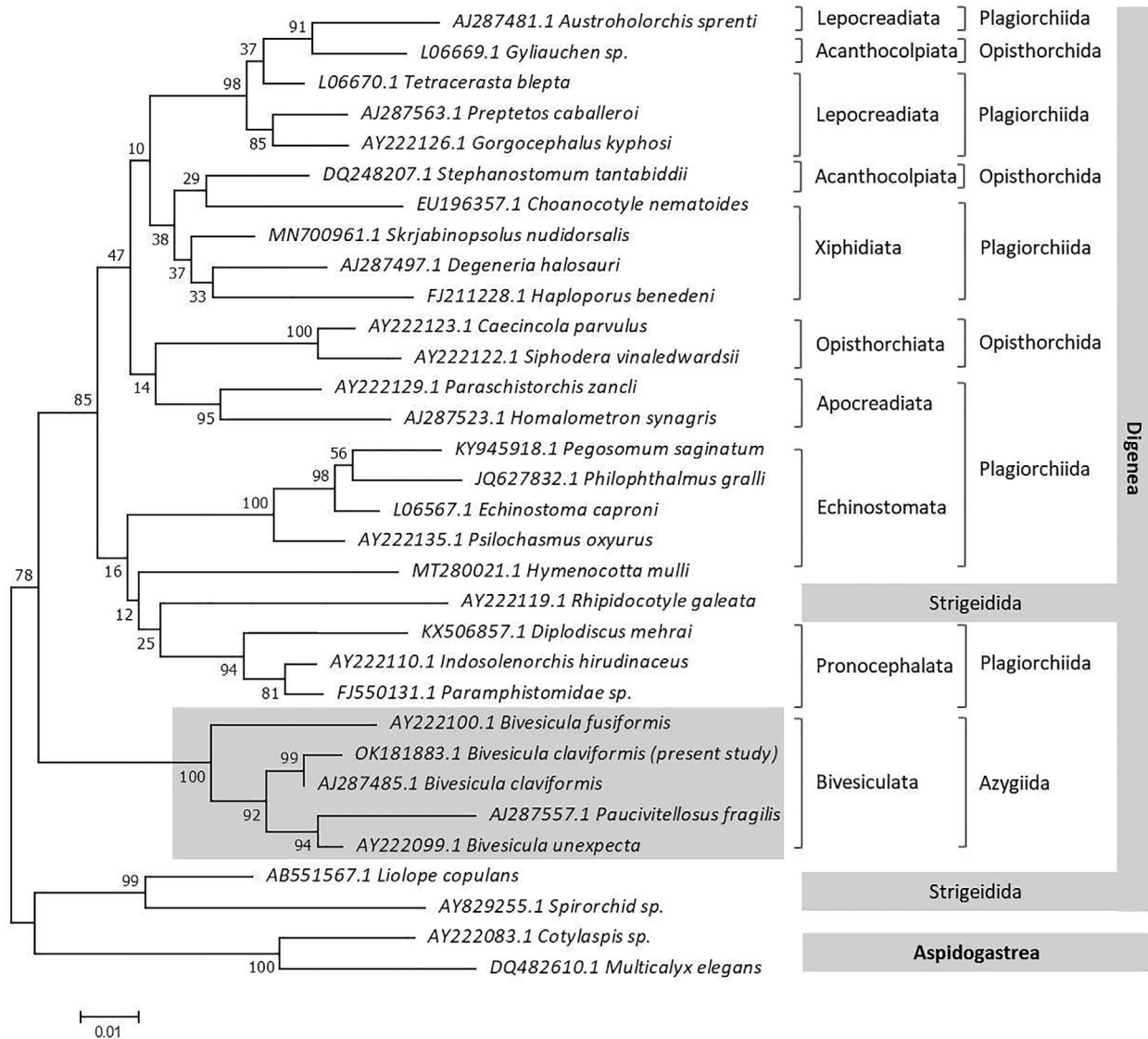


Fig. 4. Molecular Phylogenetic analysis by Maximum Likelihood method (ML) based on the Tamura-Nei model. The tree with the highest log likelihood (−6175.42) is shown.

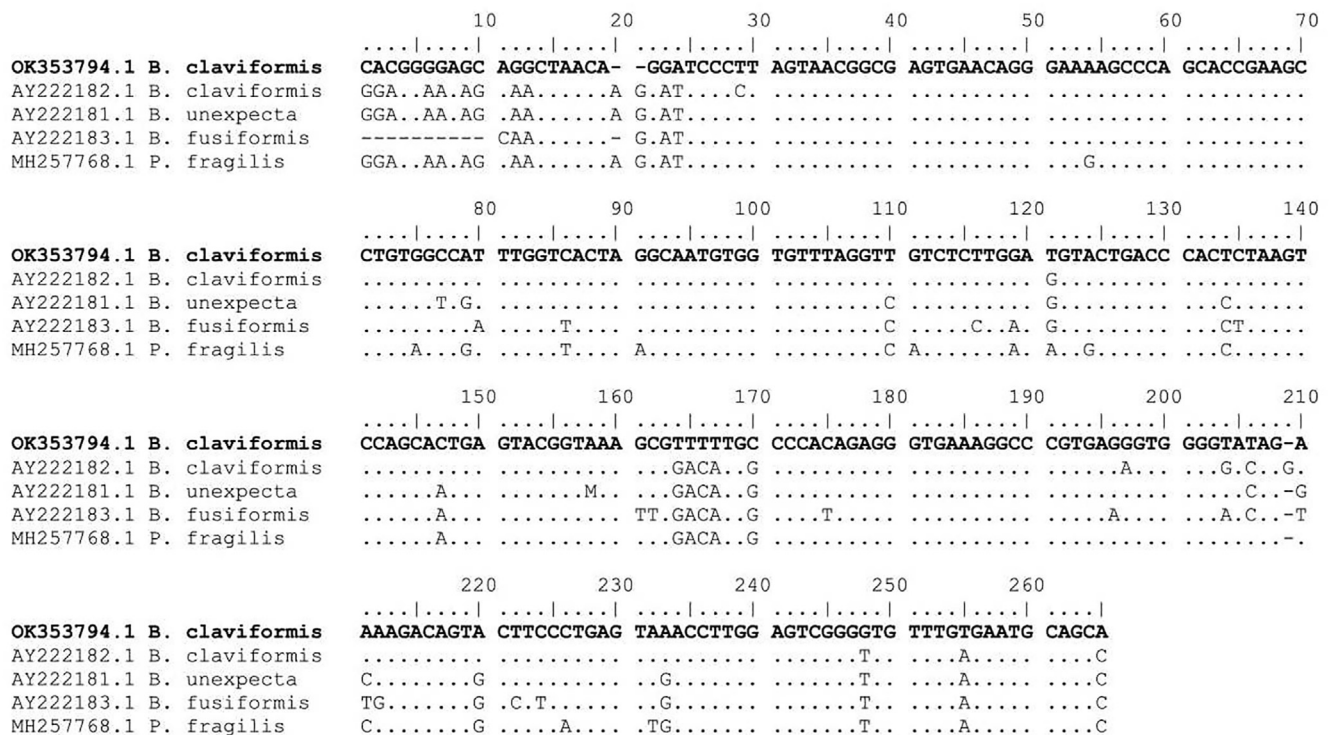
clustered two genera of Strigeidida (*AB551567.1 Liolope copulans* and *AY829255.1 Spirorchid* sp.), as well as two genera of Aspidogastrea (*AY222083.1 Cotylaspis* sp. and *DQ482610.1 Multicalyx elegans*), and the latter considered as out-group. The first major clade was split into two lineages, the 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. Haplospiranidae 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 *Paucivittellus 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 Monorchhiidae (*KJ648919.1 Pseudobacciger cheneyae* and *MN984478.1 Lasiotocus trachinoti*, respectively) within Strigeidida and Plagiorchiida, in addition to the aspidogastrea 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 3Digenea species used in the analysis of 28S rRNA sequence for *Bivesicula claviformis*.

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

**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).

Monorchidae. 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 *Paucitell-*

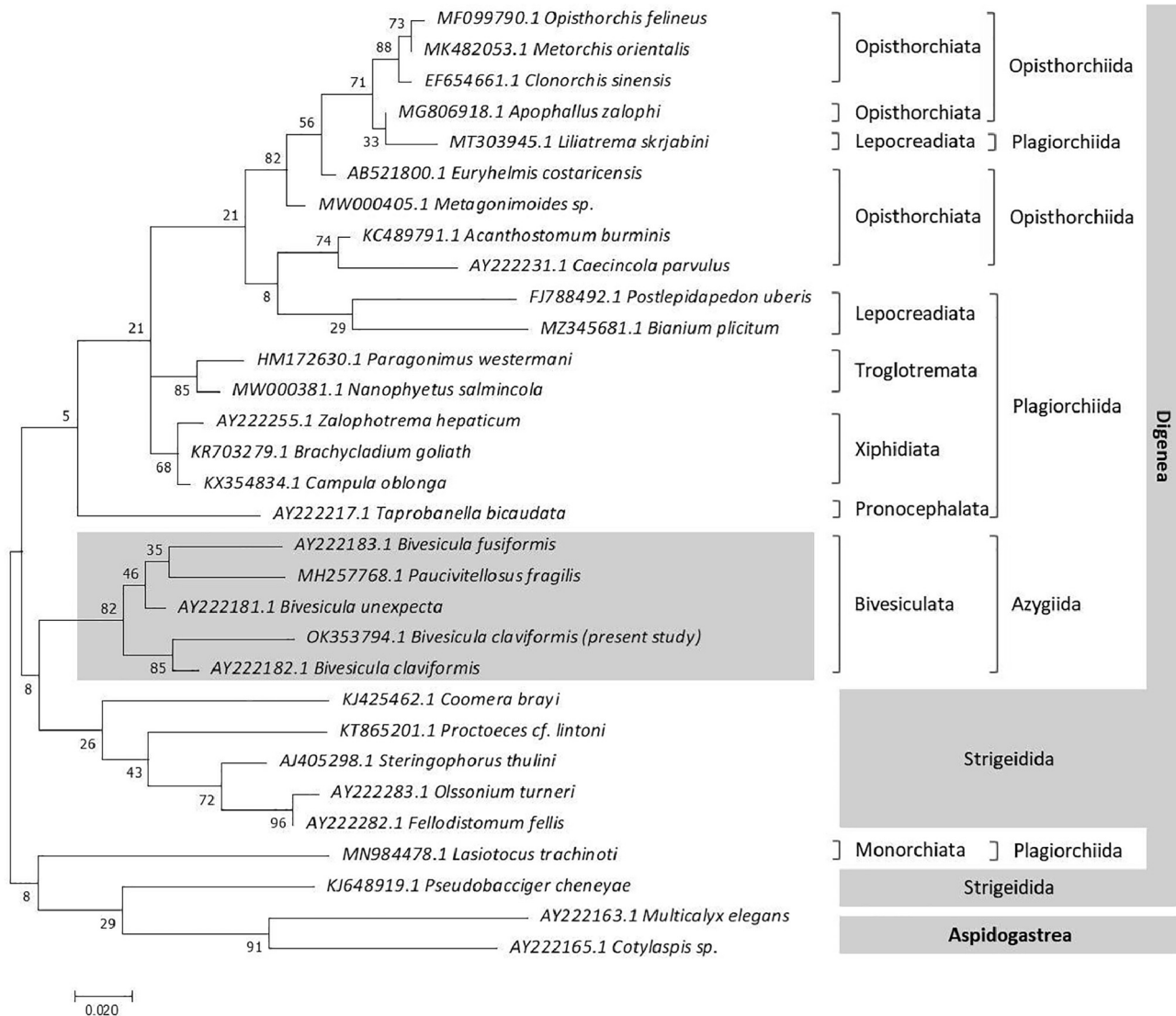


Fig. 6. Molecular Phylogenetic analysis by Maximum Likelihood method (ML) based on the Tamura-Nei model. The tree with the highest log likelihood (−1839.73) is shown.

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 Digenaea 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 bivesiculid 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 dis-

covered *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 Diplostomidae, 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.

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