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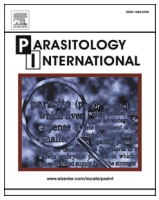

A new species of *Bivesiculoides* (Digenea: Bivesiculidae) infecting atherinid fishes of the Great Barrier Reef, Queensland, Australia

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ABSTRACT

We describe a new species of Bivesiculidae, *Bivesiculoides maiae* n. sp., from *Hypoatherina tropicalis* (Whitley) (Atherinidae) collected from off Heron Island (southern Great Barrier Reef, Queensland, Australia). *Bivesiculoides maiae* n. sp. is morphologically consistent with *Bivesiculoides* Yamaguti, 1938 in the entirely pre-testicular position of its uterus, and the possession of caeca and vitelline fields that extend posteriorly to level with the anterior extremity of the testis. The new species is morphologically distinct from the six known *Bivesiculoides* species in body size and shape, and shape of the pharynx and testis. *Bivesiculoides maiae* n. sp. is genetically distinct from the only other sequenced *Bivesiculoides* species, *Bivesiculoides fusiformis* Cribb, Bray & Barker, 1994, with which it occurs sympatrically at Heron Island. A review of related species allows two systematic recombinations. In view of the pre-testicular position of its uterus, we recombine *Bivesicula hepsetiae* Manter, 1947 as *Bivesiculoides hepsetiae* (Manter, 1947) n. comb. In view of its obtriangular body shape, round pharynx, strongly elongated testis, and the position of its ovary opposite the testis, we recombine *Bivesiculoides triangularis* Machida & Kuramochi, 2000 as *Treptodemoides triangularis* (Machida & Kuramochi, 2000) n. comb. Host-specificity of species of *Bivesiculoides* and their geographic distributions are discussed.

1. Introduction

The Bivesiculidae is one of the smaller families of trematodes infecting teleost fishes. Adult bivesiculids are morphologically distinctive in that they lack both oral and ventral suckers and possess an excretory bladder that is deeply divided into two lateral vesicles [1]. The families occupies a unique phylogenetic position in the Digenea, resolving as the basal clade for the entire Plagiorchiida [2]. The family comprises five genera: *Bivesicula* Yamaguti, 1934 (the type-genus), *Bivesiculoides* Yamaguti, 1938, *Paucivitellosus* Coil, Reid & Kuntz, 1965, *Treptodemus* Manter, 1961 and *Treptodemoides* Shen, 1995 [3]. Between the five genera there are 32 accepted bivesiculid species described worldwide [3], 28 of which have been reported from the Indo-West Pacific (IWP) region. Ten species have been reported from Australia, and of these 10, nine were reported from fishes from Queensland waters [4–8].

The genus *Bivesiculoides* is characterised by the combination of an elongated pharynx, vitelline fields extending posteriorly to the testis and an entirely pre-testicular uterus. It currently comprises six accepted species [9]. The type-species, *Bivesiculoides atherinae* Yamaguti, 1938, was described from *Hypoatherina valenciennei* (Günther) (Atheriniformes: Atherinidae) from Japan [10]. Just a single species of *Bivesiculoides*, *B. fusiformis* Cribb, Bray & Barker, 1994, has been reported from Australian waters [5]. Here we use combined morphological and molecular data to delineate two species of *Bivesiculoides* infecting atherinid fishes of Queensland, including a species new to science.

2. Materials and methods

2.1. Sample collection

Fishes were caught using seine- and hand-nets from a range of Australian locations: Heron Island (23°27′S, 151°55′E), on the southern Great Barrier Reef (GBR); Lizard Island on the northern Great Barrier Reef (14°40′S, 145°27′E); Moreton Bay (27°22′S, 153°13′E) in south-eastern Queensland; Ningaloo Reef (22°42′S, 113°40′E) in northwestern Western Australia; and off Fremantle (32°15′S, 115°41′E), in south-western Western Australia. Fishes were kept alive in tanks until

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dissection and euthanised by overdose of Aqui-S® (New Zealand Ltd.) and cranial pithing. They were identified to species level and measured using caudal fork length (lcf) as a standard measure. Trematodes were collected by whole-gut dissection and subsequent gut-wash, killed in near-boiling saline and stored in 80 % ethanol [11] at −20 °C.

2.2. Morphological analyses

Parasites were rinsed with fresh water, stained with Mayer's haematoxylin, de-stained with 1 % HCl, neutralised in 0.5 % ammonium hydroxide, dehydrated in increasing ethanol concentrations from 50 % to 100 %, cleared in 50 % and 100 % methyl salicylate (Sigma-Aldrich, Melbourne, Australia) and mounted in Canada balsam (Sigma-Aldrich, Melbourne, Australia). Only gravid adult parasites were selected for measuring and statistical analyses. For some specimens, hologenophores sensu Pleijel et al. [12] were prepared. Selected specimens were measured with OLYMPUS cellSens Standard© v1.13 on an Olympus BX53 compound microscope coupled with an Olympus SC50 camera. All measurements are in micrometres (μm) unless specified otherwise and given as a range with the mean in parentheses. Drawings were made using a camera lucida attached to the same compound microscope and digitally illustrated on Adobe Illustrator CC 2018 software. Type- and voucher specimens are lodged in the Queensland Museum (QM), Brisbane, Queensland (Australia) (QM G241284 – G241290, G241291 – G241300).

2.3. Molecular and phylogenetic analyses

Total genomic DNA was extracted from single gravid specimens with a standard phenol-chloroform extraction protocol modified from Sambrook and Russell [13]. Briefly, specimens were incubated three times: at 37 °C for >7 h in Tris-EDTA; at 55 °C for 2 h with added proteinase K (Invitrogen, Scoresby, Australia; 10 mg/mL); and at 65 °C for 10 min with added NaCl (5 M) and cetyltrimethylammonium bromide (CTAB). DNA was purified with chloroform (Merck, Melbourne, Australia) and phenol-chloroform-isoamyl alcohol (Ambion, Thebarton, Australia), precipitated with cold isopropanol (Sigma-Aldrich, Melbourne, Australia) at room temperature, washed in 70 % molecular-grade ethanol, dried, rehydrated in 25 μL of Invitrogen™ ultraPURE™ distilled water (Melbourne, Australia) at 4 °C for >7 h and stored at 20 °C.

The complete ITS2 rDNA, partial D1−D3 fragment of 28S rDNA and partial *cox*1 mtDNA gene regions were amplified with reaction solutions comprising 5 μL of 5 × Bioline MyTaq Reaction Buffer (Narellan, Australia), 0.75 μL (ITS2 and 28S) or 2 μL (*cox*1 gene) of each primer (10 μM), 0.25 μL of Bioline MyTaq™ DNA polymerase (Narellan, Australia) and 2 μL (ITS2 and *cox*1 gene, approximately 10 ng) or 4 μL (28S, approximately 20 ng) of DNA template, made to 20 μL with Invitrogen™ ultraPURE™ distilled water. The ITS2 region was amplified using primers 3S (5′-GGT ACC GGT GGA TCA CGT GGC TAG TG-3′) [14] and ITS2.2 (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′) [6] under the following denaturation-annealing-extension procedure: 1 × (3 min at 95 °C, 2 min at 45 °C, 90 s at 72 °C), 4 × (45 s at 95 °C, 45 s at 50 °C, 90 s at 72 °C), 30 × (20 s at 95 °C, 20 s at 52 °C, 90 s at 72 °C), 1 × (5 min extension at 72 °C). The 28S region was amplified using primers LSU5 (5′-TAG GTC GAC CCG CTG AAY TTA AGC A-3′) [15] and 1200R (5′-GCA TAG TTC ACC ATC TTT CGG-3′) [16] under the following denaturation-annealing-extension procedure: 1 × (4 min denaturation at 95 °C), 30 × (1 min at 95 °C, 1 min at 56 °C, 2 min at 72 °C), 1 × (1 min at 95 °C, 45 s at 55 °C, 4 min at 72 °C). The *cox*1 gene region was amplified using primers Dig_cox1Fa (5′-ATG ATW TTY TTY TTY YTD ATG CC-3′) and Dig_cox1R (5′-TCN GGR TGH CCR AAR AAY CAA AA-3′) [17] under the following denaturation-annealing-extension procedure: 1 × (3 min denaturation at 94 °C), 40 × (30 s at 94 °C, 30 s at 50 °C, 30 s at 72 °C), 1 × (10 min extension at 72 °C).

Amplicons were visualised on 1 % m/V agarose gels supplemented with 0.01 % V/V Invitrogen SYBR™ Safe (Melbourne, Australia). Sanger cycle sequencing was performed at the Australian Genome Research Facility using the same primers used for PCR amplifications, along with internal 28S primers L300F (5'-CAA GTA CCG TGA GGG AAA GTT G-3′) [18] and ECD2 (5'-CCT TGG TCC GTG TTT CAA GAC GGG-3′) [19]. Contiguous sequences were assembled with Geneious v11.1.2 (Kearse et al., 2012).

New ITS2 and *cox*1 gene data were each aligned using MUSCLE [20] in MEGA7 [21], with gap opening and extension penalties of −400 and − 100, respectively, and UPGMA clustering for iterations 1 and 2 (other parameters as default). Pairwise distance analyses were conducted for *Bivesiculoides* species in MEGA7 using the following parameters: "test of phylogeny = bootstrap", "no. of bootstrap replications = 10, 000", "model/method = p-distance", "substitutions to include = d: transitions + transversions", "rates among sites = uniform rates", and "gaps/missing data treatment = pairwise deletion".

New 28S data were aligned as above with those retrieved from GenBank (Table 1). A pairwise distance analysis was conducted for *Bivesiculoides* species in MEGA7 using the same parameters as above. The alignment was trimmed to the maximal length of the 50 % shortest sequences. In-partition gaps were removed [22] if affecting >25 % of sequences. The 28S alignment was further curated using Gblocks v.0.91b [23,24] with parameters of least-stringent selection [25]. After these procedures, 1278 bp of 28S alignment were available for phylogenetic analyses. The transition model no. 3 with gamma-distributed among-site variation (TIM3 + Γ) was selected as the best-fitting nucleotide substitution model for this dataset in jModelTest v.2.1.10 [26], using a corrected Akaike Information Criterion [27,28]. The alignment was converted in Mesquite v3.81 [29] for downstream use in Bayesian inference (BI) and Maximum Likelihood (ML) analyses in MrBayes 3.2.7a [30] and RAxML [31], respectively, accessed through the CIPRES portal [32]. The BI analyses used number of substitution types ('nst'), TIM3 model substitution rates, gamma shape fixed parameter ('shapepr') and number of rate categories for gamma distribution ('ngammacat') calculated in jModelTest2. The algorithm was run over

Table 1
28S rDNA sequence data used in the phylogenetic analyses. In bold: sequences produced in the present study.

Species	Host	28S	Reference
Bivesicula cephalopholicola	*Cephalopholis boenak*	OM459978	[8]
Bivesicula claviformis	*Epinephelus merra*	OM459982	[8]
Bivesicula gymnothoracis	*Epinephelus fasciatus*	OM459984	[8]
Bivesicula megalopis	***Megalops cyprinoides***	**PQ367906**	**This study**
Bivesicula neglecta	Apogonidae sp.	OM459986	[8]
Bivesicula novaecaledoniensis	*Epinephelus chlorostigma*	OM459987	[8]
Bivesicula obovata	*Epinephelus quoyanus*	OM459988	[8]
Bivesicula palauensis	*Epinephelus areolatus*	OM459990	[8]
Bivesicula polynesiensis	*Sargocentron caudimaculatum*	OM459993	[8]
Bivesicula unexpecta	*Acanthochromis polyacanthus*	AY222181	[2]
Bivesicula sheni	*Epinephelus maculatus*	OM459995	[8]
Bivesiculoides fusiformis	*Atherinomorus lacunosus*	AY222183	[2]
***Bivesiculoides maiae* n. sp.**	***Hypoatherina tropicalis***	**PQ367904**	**This study**
Paucivitellosus fragilis	*Clypeomorus batillariaeformis*	MH257768	[53]
Paucivitellosus vietnamensis	*Mugil cephalus*	LN831715	[54]
Treptodemoides fukenensis	***Hyporhamphus regularis***	**PQ367905**	**This study**
Outgroup			
Transversotrema hunterae	*Sillago maculata*	KX186733	[52]
Transversotrema licinum	*Acanthopagrus australis*	KX186736	[52]
Transversotrema witenbergi	*Pterocaesio marri*	KX186743	[52]

10,000,000 generations ('ngen = 10,000,000') in two runs with four Markov Chain Monte-Carlo (MCMC) chains (nchains = 4) each set at default temperature and sampled every 1000th tree (samplefreq = 1000), without discarding values for the convergence diagnostic and without early stop. Branch length information was saved. The taxa were ordered before tree printing. Tree and branch lengths were summarised using parameters 'sump burnin = 3000' and 'sumt burnin = 3000', with all compatible groups retained for consensus trees and tree probabilities printed. The ML analysis used RAxML-HPC BlackBox [31] with parameters calculated in jModelTest2 and automatic bootstrapping halt. The trees were visualised in Figtree v1.4.4 [33]. Three species of *Transversotrema* Witenberg, 1944 (Transversotrematidae) were used as outgroups for the 28S analysis. Nodal support <75 % was considered non-significant.

2.4. Species concept and delineation criteria

The Lineage Species Concept [34] was chosen for species delimitation as an effective consensus. Reciprocal monophyly of lineages was used as an obligatory species delineation criterion [34]; morphological diagnosability [35,36] and host-specificity [37] were considered as additional evidence.

3. Results

3.1. General results

Our records of the examination of marine atherinids from Australia relate to 263 individuals of three genera and six species from five localities (Table 2). Bivesiculid specimens were collected from just two of these six species, all consistent with the concept of *Bivesiculoides*. Of 54 *Atherinomorus lacunosus* (Forster) (including those leading to the original description of *B. fusiformis*), 10 were infected. Of 23 *Hypoatherina tropicalis* (Whitley), 17 were infected. All infections were from fishes collected off Heron Island.

3.1.1. Morphological analyses

All bivesiculid specimens infecting *A. lacunosus* and *H. tropicalis* were assigned to *Bivesiculoides* based on their entirely pre-testicular uterus, elongate body shape, large elongate pharynx, and caeca and vitelline fields extending posteriorly to the anterior margin of the testis [5]. The specimens collected from *A. lacunosus* from off Heron Island, the type-host and type-locality for *B. fusiformis*, were identified as that species based on their distinctly fusiform body shape, large infundibuliform pharynx, large cirrus-sac, and vitelline follicles extending from the caecal bifurcation to mid-testis. Specimens from *H. tropicalis* were not morphologically consistent with any of the currently accepted *Bivesiculoides* species.

3.1.2. Molecular analyses

Mitochondrial *cox1* gene and ribosomal ITS2 sequence data were generated for specimens of both *Bivesiculoides* taxa (Table 1). The single *cox*1 gene sequence of the uncharacterised *Bivesiculoides* taxon differed from two sequences of *B. fusiformis* at a p-distance of 19.9–20.8 %. The two *B. fusiformis* sequences differed from each other at a p-distance of 1.4 %. The two identical ITS2 sequences of the uncharacterised *Bivesiculoides* taxon differed from two identical sequences of *B. fusiformis* at a p-distance of 3.7 %.

Table 2
Numbers of atherinids collected in this study. A: Heron Island (Qld); B: Lizard Island (Qld); C: Moreton Bay (Qld); D: Ningaloo Reef (WA); E: Off Fremantle (WA).

Host Genus	Host species	A	B	C	D	E	Total
Atherinomorus	*endrachtensis*		1				1
	lacunosus	43	11				54
	vaigiensis			118	6	56	180
Craterocephalus	*mugiloides*		4				4
Hypoatherina	*barnesi*	1					1
	tropicalis	23					23
Total		67	26	118	6	56	263

Partial 28S rDNA sequences were generated for specimens of the *Bivesiculoides* taxon from *H. tropicalis* for phylogenetic analyses (Table 1). Both BI and ML phylogenetic trees showed the same topology. All sequences relating to *Bivesiculoides* formed a reciprocally monophyletic clade, strongly supported in both BI (Fig. 1) and ML (not shown) analyses and distinguishing it from species of *Bivesicula*, *Paucivitellosus* and *Treptodemoides*; *Bivesicula* resolves as paraphyletic in these analyses (Fig. 1). The uncharacterised *Bivesiculoides* taxon from *H. tropicalis* differed from the single sequence of *B. fusiformis* at a p-distance of 3.2 %.

Significant morphological and molecular differences between specimens from the two fish species suggest the presence of two *Bivesiculoides* species, where are interpreted as one known species (*B. fusiformis* from *A. lacunosus*) and one new species from *H. tropicalis*, described below.

3.2. Taxonomic synthesis

Family Bivesiculidae Yamaguti, 1934

Genus *Bivesiculoides* Yamaguti, 1934

***Bivesiculoides fusiformis* Cribb, Bray & Barker, 1994**

Type-host: *Atherinomorus lacunosus* (Forster) [reported as *Atherinomorus capricornensis* (Woodland)], Slender hardyhead (Atheriniformes: Atherinidae).

Type-locality: off Heron Island, Queensland, Australia.

Previous records: Cribb et al. [5], Olson et al. [2].

New material.

Host: *Atherinomorus lacunosus*.

Locality: off Heron Island (23°27′S, 151°55′E), southern Great Barrier Reef, Queensland, Australia.

Site in host: Intestine.

Prevalence: 10/43.

Specimens deposited: Six wholemounts and one set of serial transverse sections (QM G241284–90).

Deposition of molecular data: Two ITS2 rDNA sequences (GenBank PQ367907−08); two *cox*1 mtDNA gene sequences (GenBank PQ367883−84).

Remarks

The new specimens were comprehensively consistent with the original description of *B. fusiformis*. All the key measurements were within the ranges originally reported and the specimens are comparable and distinctive in a strongly fusiform body shape, a large infundibuliform pharynx, and in the distribution of their vitelline follicles.

***Bivesiculoides maiae* n. sp.** (Fig. 2)

Description

[Measurements based on six mature specimens.] Body fusiform, tapering towards extremities, bluntly pointed posteriorly, longer than wide, 310–452 (424) × 167–248 (209). Body length / width ratio 1.8–2.4 (2.1). Eyespot pigment scattered from anterior margin of pharynx to anterior end of cirrus-sac. Tegumental spines minute, covering entire body, longer on anterior part of body and to level of pharynx. Pharynx large, sometimes conspicuously retracted into body, ranging from bluntly V-shaped when protracted to near-spherical when retracted, rectangular with rounded edges or bluntly oval, longer than or as long as wide, 55–66 (65) × 53–82 (64), occupying 12.9–20.6 (15.3 %) of body length. Oesophagus straight to sinuous or coiled, 40–110 (82) × 8–13 (11). Caeca extend to level between anterior half and posterior end of testis. Testis singular, entire, near-spherical to irregularly ovate (wider than long in latter case), medial to dextral, in posterior half of body, 63–99 (78) × 55–79 (66). External seminal vesicle not detected. Cirrus-sac ovoid, medial, in anterior half of body, anterior margin sometimes slightly overlapping caecal bifurcation, 74–113 (95) × 55–79 (64), 1.34–1.85 (1.50) times longer than wide. Genital pore not

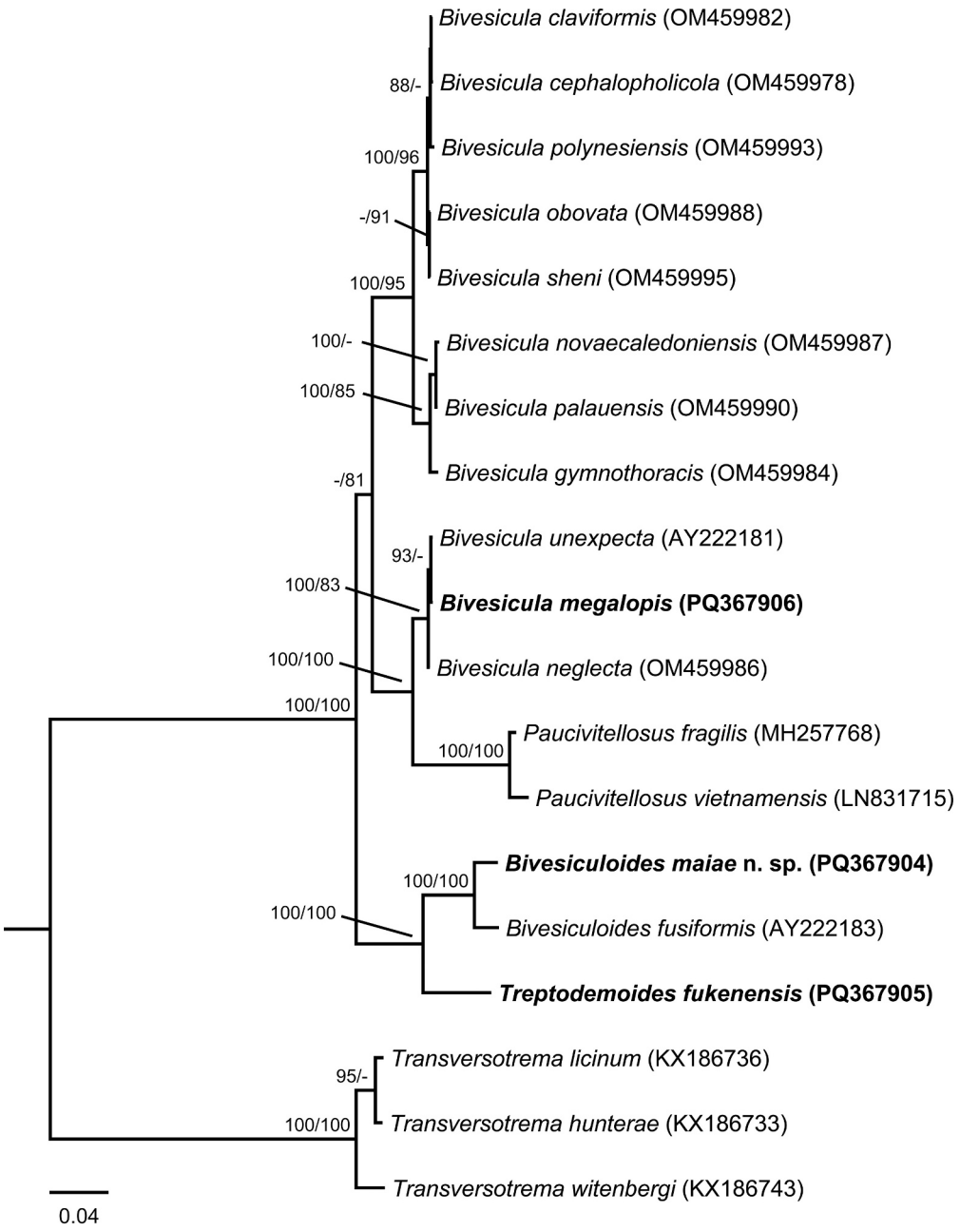

Fig. 1. Phylogenetic relationships between the Bivesiculidae generated by Bayesian Inference (BI) analysis of the partial 28S rDNA region from a 1267-bp alignment. Sequences in bold were generated in this study. Numbers above nodes are presented as 'posterior probabilities (%) / bootstrap values (%)'; only values >75 % are indicated and considered significant. The bootstrap values were calculated in the Maximum Likelihood analysis conducted on the 28S rDNA alignment (tree not shown).

observed. Internal seminal vesicle sub-spherical to ovate (wider than long in latter case), at anterior end of cirrus-sac, 24–37 (32) × 19–31 (27). Ovary bluntly ovate to rounded, dorsal, usually almost median but may be either sinistral or dextral, immediately anterior to testis, 28–50 (39) × 33–41 (35). Seminal receptacle irregularly rounded, near-medial, antero-dextral and partially ventral to testis, 43–57 (49) × 35–46 (41). Vitelline follicles irregularly globular to oblong, reaching anteriorly to level of intestinal bifurcation or sometimes to posterior margin of pharynx if retracted, rarely covering caecal bifurcation with narrow confluence, never confluent at level of cirrus-sac, tightly surrounding caeca to level near to their extremities, reaching posteriorly to posterior margin of testis in two narrow non-confluent fields. Uterus never extends posterior to testis. Eggs oval, thick-shelled, 50–67 (54) × 33–46 (40). Excretory vesicle V-shaped, ventral to caeca, surrounded by vitelline follicles, with arms extending anteriorly to caecal bifurcation where they may approach each other medially.

Taxonomic summary

Type-host: *Hypoatherina tropicalis* (Whitley) (Tropical Hardyhead) (Atheriniformes: Atherinidae).

Type-locality: off Heron Island (23°27′S, 151°55′E), southern Great Barrier Reef, Queensland, Australia.

Site in host: Intestine.

Prevalence: 17/23.

Specimens deposited: Holotype (QM G241291), five paratypes (QM G241292–296) and four immature voucher specimens (QM G241297–300).

Representative DNA sequences: One partial D1−D3 28S rDNA sequence (GenBank PQ367904); two ITS2 rDNA sequences (GenBank

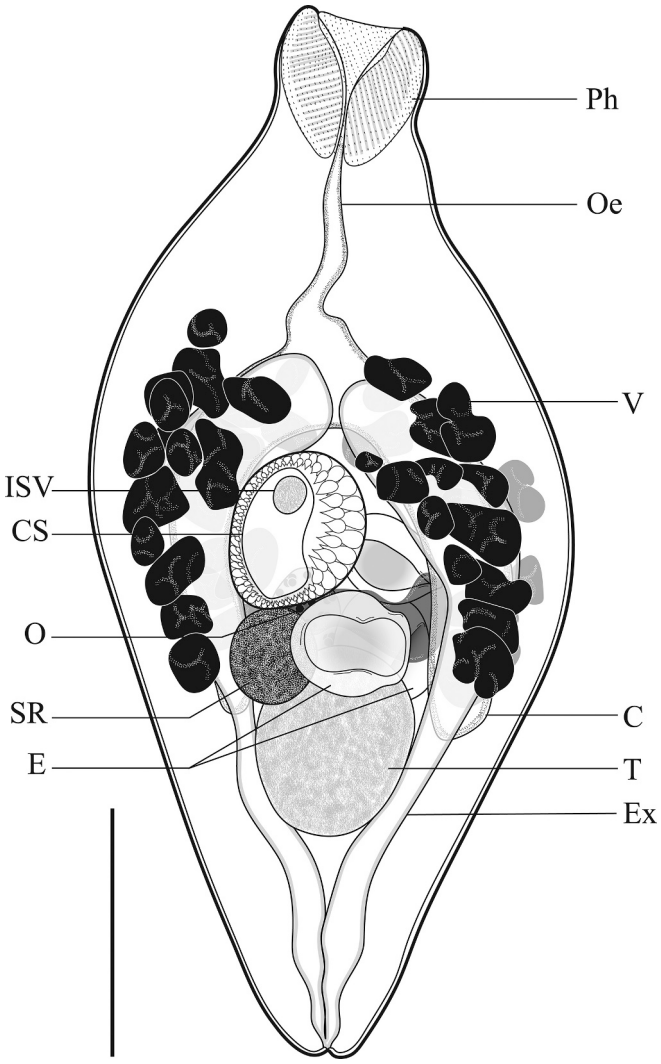

Fig. 2. *Bivesiculoides maiae* n. sp. holotype, scale: 100 μm. C, caeca; CS, cirrus sac; E, eggs; Ex, excretory vesicle; ISV, internal seminal vesicle; O, ovary; Oe, oesophagus; Ph, pharynx; SR, seminal receptacle; T, testis; V, vitelline follicles.

PQ367909−10); one *cox*1 mtDNA gene sequence (GenBank PQ367885).

ZooBank Registration: The Life Science Identifier (LSID) for *Bivesiculoides maiae* n. sp. is B305E0EF-2850-416D-9E67-01119AB7E31D.

Etymology: the Latin noun *maiae* refers to the name of the lead author's sister, Maia.

4. Discussion

4.1. Composition of Bivesiculoides

Consideration of the composition of *Bivesiculoides* leads us to transfer one previously described species in and another out of that genus. *Bivesicula hepsetiae* Manter, 1947 is consistent with *Bivesiculoides* rather than *Bivesicula* in having a uterus that extends posteriorly no further than the posterior margin of the testis [38]. Notably, it is reported from an atherinid, a host family infected by three other species of *Bivesiculoides* and not otherwise infected by species of *Bivesicula*. We therefore propose *Bivesiculoides hepsetiae* (Manter, 1947) n. comb.

Bivesiculoides triangularis Machida & Kuramochi, 2000 is here recombined as *Treptodemoides triangularis* (Machida & Kuramochi, 2000) n. comb. on the basis that it is consistent with the concept of *Treptodemoides* (see Shen [39]) rather than that of *Bivesiculoides* (see Yamaguti [10]). As such, *T. triangularis* becomes just the second presently recognised species of *Treptodemoides* after the type-species, *T. fukenensis* (Liu, 1995) Cribb, 2002. *Treptodemoides triangularis* possesses a clear inverted triangular shape whereas all species of *Bivesiculoides* are elongate [10,40,41] to fusiform [5] and ovoid [42]. Moreover, *T. triangularis* has a diagonal, sinistral, strongly elongated testis whose shape and relative position are consistent with that of *T. fukenensis* [39] and not *Bivesiculoides* [5,10,40–42]. All species of *Bivesiculoides* have the ovary anterior to the testis whereas *T. triangularis*, like *T. fukenensis*, has the ovary opposite the testis. Notably, like species of *Bivesiculoides, T. triangularis* has tegumental spines [5,10]. *Treptodemoides fukenensis* was described as spineless by Shen [39] but as having "minute spines" by Liu [43]. We think it likely that the absence of spines on *Treptodemoides* as per Shen [39] is due to improper specimen fixation or storage, as seen for two *Bivesiculoides* species [40,42]. We therefore propose that *Bivesiculoides* should be considered to comprise seven species: *B. atherinae, B. fusiformis, B. hepsetiae, B. maiae* n. sp., *Bivesiculoides otagoensis* Manter, 1954, *Bivesiculoides posterotestis* Durio & Manter, 1968 and *Bivesiculoides scari* Hafeezullah, 1971.

4.2. Differential diagnosis of Bivesiculoides maiae n. sp.

Relative to the six other currently accepted species of *Bivesiculoides*, *B. maiae* n. sp. is quite distinctive. It differs from *B. atherinae* in its smallest body size (310–452 × 167–248 μm vs 1100–2100 × 300–600 μm), its body length-egg length ratio, in having a more fusiform body shape, and in the absence of discrete eyespots. It differs from *B. fusiformis* in having a smaller (310–452 × 167–248 μm vs 821–1389 × 348–570 μm) and less fusiform body, a larger pharynx and pharynx length-body length ratio, and in having a rounder testis. *Bivesiculoides maiae* n. sp. most closely resembles *B. hepsetiae* in having the vitellarium relatively restricted and intestinal caeca extending to about the anterior margin of the testis. It differs, however, in having a fusiform body shape, a proportionally much larger pharynx and arms of the excretory vesicle that do not extend anterior to either the vitelline follicles or the intestinal bifurcation (as occurs in *B. hepsetiae*). *Bivesiculoides maiae* n. sp. is immediately distinguishable from *B. otagoensis* and *B. scari* in that both have vitelline follicles extending for most of the body length instead of for <50 % of it; indeed, the restricted vitellarium of the present species differentiates it to some extent from all six previous species. The new species differs further from *B. atherinae, B. fusiformis* and *B. posterotestis* in having a distinctly less elongate pharynx. In addition, *B. maiae* n. sp. differs from *B. otagoensis* in the extent and shape of the vitelline follicles, and in the greater distance of the testis from the posterior extremity of the body; from *B. scari* in body length (310–452 μm vs 1682–2434 μm), in egg size (50–60 × 34–45 μm vs 59–77 × 44–59 μm) and pharynx size (51–81 × 52–83 μm vs 90–113 × 111–146 μm), and in the absence of an anterior fold. The new species further differs from *B. posterotestis* in body size (364–446 × 164–245 μm vs 992–557 μm) and shape, and in a greater distance of the testis from the posterior extremity.

4.3. Host specificity and distributions

Species of the genus *Bivesiculoides* have been reported from seven fish families (Table 3). Despite this host diversity, with the proposal of *B. maiae* n. sp. from *H. tropicalis*, four of the seven species of this genus (*B. atherinae, B. fusiformis, B. hepsetiae* and *B. maiae*) infect atherinids; *Bivesiculoides* species have evidently radiated in association with atherinids to some degree. The new findings here strongly suggest that *B. fusiformis* and *B. maiae* n. sp. infect only *A. lacunosus* and *H. tropicalis*, respectively; neither species has been encountered in thousands of individuals of hundreds of other fish species examined from the same location. We therefore consider both *B. fusiformis* and *B. maiae* n. sp. as oioxenous. Other *Bivesiculoides* species infect various planktivorous (emmelichthyid and myctophid) and grazing (scarine) fishes [44,45]. It is noteworthy that *B. atherinae*, although reported initially from an atherinid, was also later reported from species of Caesionidae, Cyttidae

Table 3
Species and hosts of *Bivesiculoides*. In bold: species described in this study.

Host family	Host species	Bivesiculid species	Reference
Atherinidae	*Atherinomorus lacunosus* (Forster)	*Bivesiculoides fusiformis*	[5]
	Atherinomorus stipes (Müller & Troschel)	*Bivesiculoides hepsetiae*	[38]
	***Hypoatherina tropicalis* (Whitley)**	***Bivesiculoides maiae* n. sp.**	**This study**
	Hypoatherina valenciennei (Bleeker)	*Bivesiculoides atherinae*	[10]
Berycidae	*Beryx splendens* Lowe	*Bivesiculoides otagoensis*	[47]
Cyttidae	*Cyttus australis* (Richardson)	*Bivesiculoides atherinae*	[47]
	Cyttus traversi Hutton	*Bivesiculoides atherinae*	[47]
Caesionidae	*Dipterygonotus balteatus* Valenciennes	*Bivesiculoides atherinae*	[47]
Emmelichthyidae	*Emmelichthys nitidus* Richardson	*Bivesiculoides atherinae*	[47]
	Plagiogeneion macrolepis McCulloch	*Bivesiculoides atherinae*	[47]
	Plagiogeneion rubiginosum (Hutton)	*Bivesiculoides otagoensis*	[40,47]
Myctophidae	Unknown, reported as "shiner"	*Bivesiculoides posterotestis*	[42]
Scaridae	*Scarus ghobban* Forsskål	*Bivesiculoides scari*	[41]

and Emmelichthyidae [46,47] and *B. otagoensis* was reported from Berycidae and Emmelichthyidae [47] (Table 3). None of these subsequent reports was accompanied by figures and, in the light of the high host-specificity reported here, they require confirmation. Notably, however, recent molecular studies have shown that some species of the genus *Bivesicula* are shared between families as distantly related as holocentrids, muraenids and serranids [8]. As molecular data exist for *B. fusiformis* and *B. maiae* only, the overall validity of the present composition of the genus remains to be tested by molecular phylogenetics.

The geographic ranges of species of *Bivesiculoides* are poorly known. Each species is known from few locations, often only from the type-location: Lake Hamada, Shizuoka (Japan), the Philippine Sea and New Zealand for the type-species *B. atherinae* [10,47]; southern Great Barrier Reef for *B. fusiformis* [5]; Dry Tortugas (Florida, USA) for *B. hepsetiae* [38]; southern Great Barrier Reef for *B. maiae* n. sp.; Dunedin (New Zealand) for *B. otagoensis* [40]; Noumea (New Caledonia) for *B. posterotestis* [42]; and the Gulf of Mannar for *B. scari* [41]. This limited reporting does not preclude the presence of these species elsewhere. Indeed, with the notable exceptions of *A. stipes* and *P. macrolepis*, all the hosts of *Bivesiculoides* species are widespread in the Western Pacific and parts of the Indian Ocean [44]. Despite considerable atherinid diversity in Australia and the IWP region [44,48,49], however, only two studies record any digeneans from Australian atherinids [5,50]. Overall, the apparently restricted range of most *Bivesiculoides* species could be attributed to, either, a restricted range of their intermediate hosts, or insufficient sampling. The latter is likely given the overall lack of taxonomic effort for digeneans in much of the IWP [51]. In addition, as four of the *Bivesiculoides* species infect atherinids, each a single host species, it seems possible that the total atherinid world fauna harbours substantially greater richness for this genus. Failure to find infections in substantial samples of *A. vaigiensis* suggests, however, that not all species are infected.

CRediT authorship contribution statement

Clarisse Louvard: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Scott C. Cutmore:** Writing – review & editing, Validation, Supervision, Methodology, Investigation. **Thomas H. Cribb:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

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