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A complex of the blood fluke genus *Psettarium* (Digenea: Aporocotylidae) infecting tetraodontiform fishes of east Queensland waters

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Abstract

Seven species of *Psettarium* (Digenea: Aporocotylidae), including four new species, are reported from tetraodontiform fishes from off coastal east Queensland. *Psettarium pandora* n. sp. infects the yellow boxfish, *Ostracion cubicus* (Ostraciidae), the first known aporocotylid to infect this family of fishes. Three new species are reported from pufferfishes of the genus *Arothron* (Tetraodontidae): *Psettarium yoshidai* n. sp. infects the map puffer (*Arothron mappa*), *Psettarium hustoni* n. sp. infects the black-spotted puffer (*A. nigropunctatus*) and *Psettarium martini* n. sp. infects the starry puffer (*A. stellatus*). We also report three species of *Psettarium* from Australian waters for the first time. *Paracardicola hawaiensis* Martin, 1960, the sole species of *Paracardicola*, is redescribed based on specimens collected from the type-host, the stars-and-stripes puffer, *Arothron hispidus*. *Paracardicola* is synonymised with *Psettarium* and *P. hawaiensis* is recombined as *Psettarium hawaiiense* (Martin, 1960) n. comb. *Psettarium pulchellum* Yong, Cutmore, Bray, Miller, Semarariana, Palm & Cribb, 2016, described from the narrow-lined puffer (*Arothron manilensis*) from off Bali, Indonesia, is reported from the same fish species at two locations on the Queensland coast, significantly extending the range of this species. *Psettarium nolani* (Bray, Cribb & Littlewood, 2013), originally described from French Polynesia, is reported from *A. hispidus*, *A. manilensis* and *A. stellatus*, representing both new host and locality records for this species. Molecular phylogenetic analysis shows these species to all be closely related, such that they cannot be considered to represent separate genera despite their differing morphology. Analysis of 28S sequence data for *Psettarium anthicum* Bullard & Overstreet, 2006, a non-tetraodontiform-infecting species, shows it to be distantly related to all other species of *Psettarium* for which sequence data are available. The species is re-assigned to a new genus, *Cardallagium* n. gen., as *Cardallagium anthicum* (Bullard & Overstreet, 2006) n. comb. We think it likely that the host range of species of *Psettarium* is limited to tetraodontiform fishes. We assessed the infection biology of two species, *P. nolani* and *P. hawaiiense* n. comb. infecting *A. hispidus*, using histology to assess the pathways of egg release for these species. Eggs of both species were observed in both circulatory and visceral organs of infected hosts, often in high numbers. Eggs were seen trapped in the mucosal layer of the intestine and, in rare instances, causing lesions in the laminar epithelium, providing the strongest evidence yet that they pass through the gut wall and escape the host *via* the faeces. Lastly, we discuss the biogeographical implications of our findings, noting that some *Psettarium* species now show very wide geographical distributions.

Key words: Aporocotylidae, *Psettarium*, *Paracardicola*, Tetraodontidae, Ostraciidae, Australia

1. Introduction

Fish blood flukes (Aporocotylidae Odhner, 1912) infect the circulatory systems of a wide range of fishes from both freshwater and marine habitats worldwide. Since the description of *Aporocotyle simplex* Odhner, 1900, over 160 species have been described from 20 orders and 60 families of fishes. Eleven species have been described from fishes from the order Tetraodontiformes, of which all but one infect the pufferfish family Tetraodontidae. The primary genus, and subject of this study, is *Psettarium* Goto & Ozaki, 1930, which presently contains eight putative species known from tetraodontid fishes.

The genus *Psettarium* was established by Goto & Ozaki [1] to replace *Plehnia* Goto & Ozaki, 1929, which was preoccupied, with *Plehnia japonica* Goto & Ozaki, 1929, infecting the Japanese pufferfish species *Takifugu pardalis* (Temminck & Schlegel), renamed *Psettarium japonicum* (Goto & Ozaki, 1929) and designated as the type-species for the genus [1, 2]. The genus presently has ten species, seven of which infect pufferfishes (Tetraodontidae). Bullard & Overstreet [3] transferred two species of *Psettarioides* Lebedev & Parukhin, 1972, to *Psettarium*, neither of which infect tetraodontids. *Psettarium pseudupenei* (Lebedev & Parukhin, 1972) infects the Indian goatfish, *Parupeneus indicus* (Shaw) (Perciformes: Mullidae) and *P. rachycentri* (Lebedev & Parukhin, 1972) infects the cobia, *Rachycentron canadum* Linnaeus (Perciformes: Rachycentridae) [4]. Bullard & Overstreet [3] also described a further species, *P. anthicum* Bullard & Overstreet, 2006, from *R. canadum*.

The pufferfish genus *Arothron* Müller has been shown to harbour a rich aporocotylid fauna. Martin [5] described *Paracardicola hawaiensis* Martin, 1960, from the stars-and-stripes puffer, *Arothron hispidus* (Linnaeus) (as *Tetraodon hispidus*), from off Hawaii. Yong & Cribb [6] described *Rhaphidotrema kiatkiongi* Yong & Cribb, 2011, from the same host species off Lizard Island, on the far north Great Barrier Reef (GBR). Bray *et al.* [7] described *S. nolani* (now *Psettarium nolani*) from the guineafowl puffer, *A. meleagris* (Anonymous), from French Polynesia. The three species described by Yong *et al.* [8] from Bali infect the reticulated puffer, *Arothron reticularis* (Bloch & Schneider), and the narrow-lined puffer, *A. manilensis* (de Procé). Our examinations of species of *Arothron* in eastern Australian waters have revealed further instances of infection among these fishes, including several novel species, new hosts and new locality records. Our investigations also reveal a new aporocotylid species infecting a member of the boxfish family Ostraciidae, the first known from this family of fishes. Here, we characterise these taxa, all recognised as species of *Psettarium*, using both morphological and molecular phylogenetic methods.

Most aporocotylid species primarily infect the heart and gills of their hosts, but some tetraodontid-infecting species of *Psettarium* are notable in primarily infecting the mesenteric vessels. Of the seven known species, four [*P. japonicum*, *P. nolani*, *P. sinense* (Liu, 1997) and *P. tropicum* (Manter, 1940)] are known or presumed to infect this site. Two other tetraodontid-infecting species, *Paradeontacylix odhneri* (Layman, 1930) and *Paracardicola hawaiensis*, also infect mesenteric vessels. The eggs of *P. hawaiensis* and *P. nolani* were noted to be accumulating in visceral organs, the liver in the case of *P. hawaiensis* and the intestinal wall in *P. nolani* [5, 7]. Eggs of *P. sinense*, as well as those of the putative *Psettarium* species from aquacultured Japanese tiger puffer, *Takifugu rubripes* (Temminck & Schlegel), have also been noted in visceral organs of infected hosts, causing significant pathology and mortality in aquacultured puffer stocks [9-12]. The standard life-cycle paradigm of aporocotylids involves eggs released into the bloodstream and travelling to the gills, where the miracidial larvae hatch and exit the host. While it has been speculated that the host gut offers an alternative means of egg or larval escape,

particularly for mesentery-infecting species like *P. sinense* [9, 12], this method has yet to be conclusively demonstrated. We therefore sought to undertake histological analysis of infected pufferfish guts, in order to investigate this alternative pathway of egg escape.

2. Materials and Methods

2.1 Specimen collection

Pufferfishes and boxfishes were obtained from locations along the eastern Queensland coast using a variety of methods, including spears, barrier nets, seine nets, commercial tunnel nets and hand lines. The heart, branchial arteries, gills and mesenteries were examined for worms, while squashes of gill and heart tissue were examined for the presence of eggs following Yong *et al.* [13]. Aporocotylids were heat-fixed in near-boiling saline and stored in 70% ethanol. In some cases, egg-bearing gill tissue was preserved in 80% ethanol, while mesenteries and organs bearing trapped eggs were fixed in Bouin's fixative and preserved in 70% ethanol.

2.2 Morphological analysis

Specimens for morphological analysis were washed in fresh water and stained with Mayer's haematoxylin, de-stained with 1% HCl, neutralised in 1% ammonia solution and dehydrated in a graded ethanol series. Specimens were cleared in methyl salicylate and mounted in Canada balsam. Drawings were made with the aid of a camera lucida mounted on an Olympus BX-53 light microscope, and digitised using Adobe Illustrator version 6.0 (Adobe). Measurements were made using an Olympus SC50 digital camera attachment mounted on an Olympus BX-53 compound microscope and cellSens™ version 1.13 (Olympus) imaging software. All measurements are in micrometres (µm) and given as a range with the mean in parentheses. Where breadth follows length, the two measurements are separated by '×'. The type- and voucher specimens are deposited in the Queensland Museum, Brisbane, Australia (QM).

2.3 Histopathological analysis

Observations of gross pathology affecting gills and various visceral organs, including the liver, spleen, kidney, intestine and mesenteric tissues, were made at the time of dissections. Wet mounts of squashed tissue from infected organs were observed and photographed under a compound microscope. Care was taken to isolate and dissect each organ separately to avoid cross-contamination of tissues. In three cases of especially heavily-infected fishes (all *Arothron hispidus*), whole opened and un-opened intestines were preserved for histological analysis. Dissected intestines were cut lengthwise and wrapped flat around wooden dowels before being left in Bouin's fixative overnight, as per Wood & Bacha [14]. Whole unopened intestines were also fixed overnight in Bouin's fixative, with the mesenteric vessels having been removed. Fixed intestines were then washed to remove excess fixative and stored in 70% ethanol. For sectioning, standard paraffin wax sectioning techniques were used. Fixed tissue was embedded in Paraplast Plus (Leica Biosystems, Melbourne, Australia). Ten µm serial sections were taken, stained using both standard eosin/haematoxylin and Masson's trichrome and mounted on glass slides in DePeX mounting medium (Sigma-Aldrich/Merck, Darmstadt, Germany).

2.4 Molecular analysis

Total genomic DNA was extracted using standard phenol/chloroform extraction techniques [15]. Partial 28S nuclear ribosomal DNA was amplified using the primers LSU5 (5'-TAG GTC GAC CCG CTG AAY TTA AGC A-3') [16] and either 1200R (5'-GCA TAG TTC ACC ATC TTT CGG-3') [17] or 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3') [18] and the ITS2 region using the primers 3S (5'-GGT ACC GGT GGA TCA CGT GGC TAG TG-3') [19] and ITS2.2 (5'-CCT GGT TAG TTT CTT TTC CTC CGC-3') [20]. PCR for both 28S and ITS2 regions was performed with a total volume of 20 µl consisting of approximately 10 ng of DNA, 5 µl of 5× MyTaq Reaction Buffer (Bioline), 0.75 µl of each primer (10 µM) and 0.25 µl of Taq DNA polymerase (Bioline MyTaq™ DNA Polymerase), made up to 20 µl with Invitrogen™ ultraPURE™ distilled water. Amplification was carried out on a MJ Research PTC-150 thermocycler. Amplification of the ITS2 region was carried out using the following profile: an initial single cycle of denaturation for 3 min at 95°C, annealing for 2 min at 45°C, extension for 90 s at 72°C, followed by 4 cycles of denaturation at 95°C for 45 s, annealing for 45 s at 50°C, extension for 90 s at 72°C, followed by 30 cycles of denaturation for 20 s at 95°C, annealing for 20 s at 52°C, extension for 90 s at 72°C, and a final extension for 5 min at 72°C. Amplification of the 28S region was carried out using the following profile: an initial denaturation for 4 min at 95°C, followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 56°C, extension for 2 min at 72°C, followed by a single cycle of denaturation for 1 min at 95°C, annealing for 45 s at 55°C and a final extension for 4 min at 72°C. Amplified DNA was purified using a Bioline ISOLATE II PCR and Gel Kit, according to the manufacturer's protocol. Cycle sequencing of purified DNA was carried out using the same primers used for PCR amplification as well as the additional 28S primers 300F (5'-CAA GTA CCG TGA GGG AAA GTT G-3') [21] and ECD2 (5'-CCT TGG TCC GTG TTT CAA GAC GGG-3') [22], and the additional ITS2 primer GA1 (5'-AGA ACA TCG ACA TCT TGA AC-3') [23]. Cycle sequencing was carried out at the Australian Genome Research Facility. Sequencher™ version 4.5 (GeneCodes Corp.) was used to assemble and edit contiguous sequences.

2.5 Molecular phylogenetic analysis

The ITS2 and partial 28S rDNA sequences generated in this study (Table 1) were aligned with sequence data for other species of Aporocotylidae available on GenBank (Table 2). Species of the Spirorchiidae were designated as the functional outgroup for analyses of the partial 28S rDNA region, whereas *Skoulekia meningialis* was used as the outgroup taxon for analyses of the ITS2 rDNA region. Alignments were performed using MUSCLE 3.7 [24] with ClustalW sequence weighting and UPGMA clustering for iterations 1 and 2. The resultant alignments were refined by eye using MESQUITE v3.2 [25], with the ends trimmed to match the shortest sequence in the alignments. Bayesian inference and Maximum Likelihood analyses of both the ITS2 and partial 28S rDNA datasets were conducted to explore relationships between these taxa. Bayesian inference analyses were conducted using MrBayes v3.2.6 [26], run on the CIPRES portal [27]. Maximum Likelihood analyses were conducted using RAxML v8.2.6 [28] run on the CIPRES portal. jModelTest v2.1.5 [29] was used to estimate the best nucleotide substitution model for the dataset. The TVM+I+Γ and GTR+Γ models were predicted as the best estimators for the 28S and ITS2 datasets respectively by the Akaike Information Criterion (AIC) in jModelTest, with the closest approximation of these models implemented in the Maximum Likelihood analyses. Bayesian inference analysis was run over 10,000,000 generations (ngen = 10,000,000) with two

runs each containing four simultaneous Markov Chain Monte Carlo (MCMC) chains (nchains = 4) and every 1,000th tree saved (samplefreq = 1,000). Bayesian inference analysis used the following parameters: nst = 6, rates = invgamma (for 28S) or gamma (for ITS2), ngammacat = 4, and the priors parameters of the combined dataset were set to ratepr = variable. Samples of substitution model parameters, and tree and branch lengths were summarised using the parameters 'sump burnin = 3,000' and 'sumt burnin = 3,000'. Nodal support in the Maximum Likelihood analysis was estimated by performing 100 bootstrap pseudoreplicates.

3. Results

Psettarium **Goto & Ozaki, 1930**

Syns. *Plehnia* Goto & Ozaki, 1929 (preoccupied); *Paracardicola* Martin, 1960 (new synonym); *Psettarioides* Lebedev & Parukhin, 1972

3.1 Aporocotylidae from boxfishes (Tetraodontiformes: Ostraciidae)

3.1.1 Psettarium pandora **n. sp.** **Fig 1A, 2A**

Description: [Based on 18 whole-mounted specimens]. Body broadly lanceolate to sublingual, ventrally concave, with distinct protuberance at male genital pore, 1867–2281 × 396–530 (2039 × 464), 3.7–4.7 (4.4) times longer than broad. Tegumental spines minute, straight, less than 1 long, arranged in marginal transverse rows that wrap dorsoventrally; rows spaced 2 apart throughout, begin just posterior to oral sucker, initially 9 in length and bearing indeterminate number of spines, increasing to 33–35 (34) in midbody and bearing 18–22 (20) spines each, then decreasing to 11 – 12 (11) at posterior extremity with spine count decreasing to indeterminate number. Nerve cords run length of body, 67–113 (89) from body margins, well-defined. Dorsal nerve commissure 112–172 (149) from anterior extremity, 52–91 (66) across. Oral sucker generally distinct but not detected in several specimens, apparently retractable, aspinous, 15–28 × 32–47 (22 × 41). Mouth a simple pore, terminal to just ventrally subterminal, to 9 from anterior extremity. Oesophagus straight, occupying 453–650 (553), 24.2–31.0% (26.9%) of total body length. Intestinal bifurcation medial in anterior half of body; caeca form X-shape. Anterior caeca much shorter than posterior caeca, sinuous with prominent diverticula, subequal in length. Left anterior caecum 95–164 (133); right anterior caecum 104–211 (148). Posterior caeca sinuous with prominent diverticula, subequal to greatly unequal in length with longer caecum terminating close to anterior margin of ovary. Left posterior caecum 624–1049 (849); right posterior caecum 726–1127 (877). Posterior caeca 4–9 (7) times longer than anterior caeca. Total caecal length occupying 47–58% (52%) of total body length.

Testis single, massive, with irregularly lobed margins, intercaecal, extending anteriorly to level of intestinal bifurcation medially, with lobes extending beyond to occupy spaces between diverticula of anterior caeca, laterally beyond caecal margins and nerve cords, posteriorly to ovary, 827–1307 × 128–203 (1134 × 172). Vas deferens originating medially from posterior margin of testis, passing postero-sinistrally between ovarian lobes and under uterine bend before entering cirrus-sac. External seminal

vesicle and posterior testis absent. Cirrus-sac elongate reniform, accommodating loosely-coiled duct, 74 × 36. Male genital pore dorso-sinistral, raised from body and opening terminally on prominent dorsally-directed protuberance, opening 30 – 55 (42) from lateral margin and 152–255 (196) from posterior extremity.

Ovary prominently bilobed with highly lobed surface, immediately posterior to testis, medial to submedial, 87–154 × 143–208 (117 × 172). Oviduct originates medially from posterior margin of ovary, passes posteriorly parallel to dextral body margin, then anteriorly to oötype. Oötype distinct, well-defined. Uterus tightly coiled in regular loops, passing anteriorly almost to posterior ovarian margin, then posteriorly to open at female genital pore. Female genital pore dorso-sinistral, anterior and dextral to cirrus-sac and male genital pore, 53–120 (96) from body margin, 251–360 (303) from posterior extremity. Vitelline follicles extensive, dense, evenly distributed from near anterior extremity to level of female genital pore, extending laterally beyond nerve cords almost to body margins. Vitelline duct runs medially, traceable from near caecal bifurcation to oötype, dorsal to rest of reproductive organs. Eggs seen trapped in gills; not measured.

Excretory system indistinct. Excretory pore terminal.

3.1.2 Taxonomic summary

Type-host: *Ostracion cubicus* Linnaeus, Yellow boxfish (Tetraodontiformes: Ostraciidae).

Type-locality: North Wistari Reef, off Heron Island (23°27'S, 151°52'E).

Site in host: Gill arches, heart and branchial vessels. Trapped eggs seen in heart tissue and gill lamellae.

Prevalence: Heron/Wistari Reef - Total prevalence 18 of 24 fish (75%). 13 fish with 1–6 worms (ave. 3 worms per fish), 5 fish with only eggs detected. None of two fish from Gold Coast, six from Lizard Island or three from Moreton Bay infected.

Type-material: Holotype (QM XX) and 17 paratypes (QM G XX).

Representative DNA sequences: ITS2 rDNA, three identical replicates (one sequence submitted: GenBank XX); partial 28S rDNA, two identical replicates (one sequence submitted: GenBank XX).

Etymology: The species name "*pandora*" is an allusion to the opening of Pandora's Box of Greek mythology, in reference to the discovery of this species in a boxfish.

3.1.3 Remarks

Psettarium pandora n. sp. is consistent with the genus *Psettarium*, most notably in possessing a lanceolate-to-sublingual body, X-shaped caeca, a large and mostly intercaecal anterior testis, a lobed post-testicular ovary, and an oviduct and vitelline duct dextral to the rest of the uterine and male genital complexes. It can be distinguished from four species of *Psettarium* (*P. jimbaranense* Yong, Cutmore, Bray, Miller, Semarariana, Palm & Cribb, 2016, *P. nolani*, *P. ogawai* Yong, Cutmore, Bray, Miller, Semarariana, Palm & Cribb, 2016 and *P. pulchellum* Yong, Cutmore, Bray, Miller, Semarariana, Palm & Cribb, 2016) in lacking a posterior testis entirely. In having a single large medial testis that extends

anteriorly to the level of the anterior caeca, posteriorly exceeding the extremities of the posterior caeca, and laterally beyond the margins of the nerve cords, this species most closely resembles *P. japonicum*, *P. pseudupenei*, *P. rachycentri*, *P. sinense* and *P. tropicum*. Of these five, *P. pandora* n. sp. can be immediately distinguished from *P. pseudupenei* in having a much higher ratio of anterior caecal length to posterior caeca (1:7 as opposed to 1:1.7); in addition, the host of the latter species is dramatically different – the goatfish *Parupeneus indicus* (Perciformes: Mullidae). The caeca of *P. pandora* n. sp. terminate close to the anterior margin of the ovary; this differentiates it from *P. japonicum*, *P. sinense* and *P. tropicum*. Lastly, *P. pandora* n. sp. is distinguished from *P. rachycentri* in not having dendritic, posteriorly-directed lateral projections on its posterior caeca, a dextrally-directed female genital pore positioned medially in the body, and the male genital pore on the body margin, posterior to the rest of both genital complexes. Like *P. pseudupenei*, the host for *P. rachycentri* is also very different from that of *P. pandora* n. sp.; it infects the cobia, *Rachycentron canadum*, a large pelagic perciform in a monotypic family. *Psettarium pandora* n. sp. differs from all species of *Psettarium* for which molecular data are known by 19–26 bp in the ITS2 rDNA region and 39–46 bp in the partial D1–D3 28S region sequenced (Table 3). Notably, the maximum number of differences is somewhat higher than observed between tetraodontid-infecting species (see descriptions and discussion below).

3.2 Aporocotylidae from pufferfishes (Tetraodontiformes: Tetraodontidae)

***3.2.1 Psettarium hawaiiense* (Martin, 1960) n. comb.**

new syn. *Paracardicola hawaiiensis* Martin, 1960 **Fig 3, 4A**

Description: [Based on 21 specimens, including 1 hologenophore]: Body narrowly lanceolate, broadest at level of testis or, if turgid, caecal bifurcation, 702–2228 × 55–206 (1366 × 119), 9–15.4 (11.8) times longer than broad. Tegumental spines straight, 3 long, arranged in marginal transverse rows that wrap dorsoventrally; first row immediately at base of oral sucker and last rows 16–44 (28) from excretory pore. Rows spaced 3 apart, 6–7 long throughout, bearing 8 spines each. Nerve cords well-defined, run length of body 9–35 (18) from body margins. Dorsal nerve commissure 44–136 (91) from anterior extremity, 17–47 (31) across. Oral sucker well defined, nearly spherical, 18–39 × 18–40 (24 × 29), bearing five concentric rows of fine spines. Mouth a simple pore, terminal to just ventrally subterminal. Oesophagus mostly straight, gently sinuous in some specimens, 267–651 (441), occupying 27.5–36.2% (33.0%) of total body length. Intestinal bifurcation medial in anterior half of body; caeca X-shaped. Anterior caeca straight, equal to subequal in length. Left anterior caecum 36–105 (76); right anterior caecum 42–118 (70). Posterior caeca straight with minimal diverticulation, with posterior extremities swollen, equal to subequal in length, run tightly parallel to one another. Left posterior caecum 178–459 (354); right posterior caecum 163–497 (350). Posterior caeca 4.4–8.5 (5.8) × longer than anterior caeca; total caecal length occupying 28.7–37.4% (33.5%) of total body length.

Anterior testis irregularly oblong, margins not lobed, extends laterally to exceed lateral nerve cords, anteriorly to occupy space left by shorter posterior caecum, 83–482 × 30–153 (161 × 82). Vas deferens originates medially from posterior margin of testicular field, passes under uterine coils and bifurcates just posterior to cirrus-sac, one duct leading to cirrus-sac and other to posterior testis. Posterior testis

triangular, well-developed, occupying most of post-uterine space, posterior to remainder of genitalia, 95–334 × 23–120 (165 × 55). Seminal vesicle reniform, reflexed, with indistinct internal duct variably sperm-filled, 25–156 × 18–67 (85 × 40). Male genital pore dorso-sinistral, sometimes raised from body on dorsally-directed protuberance 6–23 (18) in length, opening 6–18 (12) from lateral body margin, 149–514 (319) from posterior extremity.

Ovary oblong, margins unlobed in most specimens, lobed in some, medial, entirely post-testicular, 17–45 × 26–110 (32 × 63). Oviduct originates dextrally from posterior margin of ovary, passes medially and posteriorly, then anteriorly to meet oötype. Oötype well-defined, medial or nearly so in body, 122–393 (229) from posterior extremity. Uterus not strongly convolute, passing anteriorly to posterior ovarian margin, then posteriorly to meet female genital pore. Female genital pore dorso-sinistral, anterior and dextral to cirrus-sac and male genital pore, 7–54 (23) from body margin, 154–595 (348) from posterior extremity. Eggs seen *in utero* as well as trapped in host tissues; teardrop-shaped, distinctly mucronate at one end, 87–109 × 33–45 (104 × 41) (Fig. 3C). Vitelline follicles irregularly bundled, not confined by nerve cords, extend from dorsal nerve commissure to level of female genital pore. Vitelline duct runs medially from near caecal bifurcation to oötype, dorsal to remainder of genitalia.

Excretory vesicle 12–25 × 4–8 (19 × 5), with paired collecting ducts extending anteriorly; terminus indeterminate. Excretory pore terminal.

3.2.2 Taxonomic summary

Type-host: *Arothron hispidus* (Linnaeus), Stars-and-stripes puffer (Tetraodontiformes: Tetraodontidae).

Type-locality: Kaneohe Bay, Oahu, Hawaii, USA, central north Pacific (21°25'N, 157°47'W).

New localities: Moreton Bay region, southeast Queensland, Australia (27°23'S, 153°19'E).

Gold Coast Seaway, Gold Coast, southeast Queensland, Australia (27°56'S, 153°24'E).

Mackay Marina, Mackay, north Queensland, Australia (21°06'S, 149°13'E).

Material: Voucher specimens (QMXX) including 1 hologenophore (QMXX). All specimens ex *A. hispidus*.

Site in host: Primarily mesenteric vessels. Trapped eggs found in intestinal wall, liver, spleen, kidney and on mesenteric connective tissues, rarely in gill filaments.

Prevalence: Gold Coast – Four of six fish infected with 1–12 worms, all as concurrent infections with *P. nolani*. Mackay – One of one fish infected with 20 worms. Moreton Bay – Five of 13 fish infected with 1–5 worms, three as concurrent infections with *P. nolani*. None of five fish infected from Lizard Island.

Representative DNA sequences: ITS2 rDNA, two identical replicates, one from off Mackay and one from Moreton Bay (one sequence submitted: GenBank XX); partial 28S rDNA, two identical replicates (one sequence submitted: GenBank XX).

3.2.3 Remarks

This species was found infecting specimens of *Arothron hispidus* collected from three localities along the east Queensland coast. The specimens closely resemble the original description of *Paracardicola hawaiensis*, as described from the same host species from Hawaii. This species has not been recorded since its original discovery and description by Martin (1960). As the type-series of *P. hawaiensis* is considered lost [30], we could not compare our specimens with those of Martin and can only rely on the illustrations provided in the original description. We note some minor differences between our specimens and that illustrated by Martin, most notably in the position of the oötype and the morphology of the seminal vesicle and genital pores. The öotype was not characterised by Martin, but the juncture of the vitelline duct and uterus was illustrated as being immediately posterior to the ovary, implying that the öotype is located somewhere along the proximal descending section of the oviduct. We suspect this is incorrect; in all our specimens, the öotype is on the distal ascending section of the oviduct. We did not observe the "raised group of cells surrounding the female pore" noted by Martin, nor did the genital pores open marginally on any of our specimens, as they are depicted in Martin's illustration (see Plate 1, Fig. 3 of that paper). Despite these differences, our specimens otherwise conform closely to *P. hawaiensis* and, in the absence of further morphological or molecular data from the type-locality, are best interpreted as that species. Molecular analysis of both ITS2 and partial 28S rDNA regions shows *P. hawaiensis* belonging to the clade formed by other species of *Psettarium* which infect pufferfishes of the genus *Arothron* (see Discussion). Noting this, and the fact that the morphological features described by Martin are now consistent with the concept of *Psettarium*, we propose to re-combine the sole species of *Paracardicola* with *Psettarium* as *Psettarium hawaiiense* n. comb. Specimens from southeast Queensland and Mackay were identical in the ITS2 rDNA region. Specimens of *P. hawaiiense* n. comb. were encountered in the mesenteric vessels, their presence often indicated by large accumulations of eggs on the visceral surfaces and in organs such as the liver and spleen. The morphology of these eggs matched those described by Martin (Figs 3C, 4A).

***3.2.4 Psettarium hustoni* n. sp.** **Fig. 1B, 2B, 4B, 5**

Description: [Based on 5 whole-mounted specimens, including one hologenophore (posterior end only)]. Body narrowly lanceolate with margins narrowing at level of female genital pore, then broadening, creating paddle-like shape to posterior end; body dorsally convex, with distinct dorsal 'hump' created by cirrus-sac, 2326–2904 × 197–333 (2650 × 286), 7.5–12.5 (9.3) times longer than broad. Tegumental spines straight, 3 long. Spines arranged in marginal transverse rows that wrap dorsoventrally; first row 5–9 (7) from base of oral sucker, last row 0–24 (13) from excretory pore. Rows spaced 3–4 apart, 6–8 long throughout, bearing 7–8 spines each. Nerve cords well-defined, run length of body 31–55 (42) from body margins. Dorsal nerve commissure 85–126 (104) from anterior extremity, 58–88 (70) across. Oral sucker well defined, 25–30 × 36–42 (27 × 39). Mouth a simple pore, terminal to just ventrally subterminal. Oesophagus mostly straight, gently sinuous in some specimens, 605–811 (701), 24.5–28% (26.5%) of total body length. Caeca X-shaped; intestinal bifurcation medial in anterior half of body. Anterior caeca straight with minimal diverticulation, equal to subequal in length. Left anterior caecum 131–225 (179); right anterior caecum 128–230 (171). Posterior caeca straight with minimal diverticulation, posterior extremities swollen, equal to subequal in length, run tightly parallel to one another. Left posterior caecum 417–571 (498); right posterior caecum 355–669 (486). Posterior caeca

3.1–4.2 (3.7) × longer than anterior caeca; total caecal length occupying 24.5–31.0% (29.0%) of total body length.

Anterior testes number 11–15 (12), mainly arranged in opposite pairs, entirely post-caecal. Individual testes irregularly spherical to cuboid, margins lobed, 50–172 × 67–140 (108 × 93). Testicular field exceeds lateral nerve cords; total area 613–908 × 175–287 (732 × 235). Vas deferens originates medially from posterior margin of testicular field, passes under uterine coils and bifurcates just posterior to cirrus-sac, one duct leading to cirrus-sac and other to posterior testis. Posterior testis distinctly and irregularly lobed well-developed, occupying most of post-uterine space, posterior to remainder of genitalia, 129–271 × 134–185 (225 × 159). Cirrus-sac reniform, mainly filled by mass of glandular cells surrounding broad, relatively indistinct duct, 119–143 × 47–79 (130 × 65). Male genital pore dorso-sinistral, raised from body on dorsally-directed protuberance, opening 34–45 (37) from lateral body margin, 344–494 (434) from posterior extremity.

Ovary oblong, margins unlobed, medial, entirely post-testicular, 83–130 × 114–206 (102 × 160). Oviduct originates dextrally from posterior margin of ovary, passes medially and posteriorly, then anteriorly to meet oötype. Oötype well-defined, medial or nearly so, 255–359 (306) from posterior extremity. Uterus slightly to distinctly coiled, passing anteriorly almost to posterior ovarian margin, then posteriorly to meet female genital pore. Female genital pore dorso-sinistral, anterior and dextral to cirrus-sac and male genital pore, 39–61 (53) from body margin, 351–535 (472) from posterior extremity. Eggs seen in gills and *in utero*, spheroidal, 32–42 × 22–32 (40 × 28). Vitelline follicles irregularly bundled, not confined by nerve cords, extend from dorsal nerve commissure to level of female ge nital pore. Vitelline duct runs medially from near caecal bifurcation to oötype, dorsal to remainder of genitalia.

Excretory vesicle 25–33 × 7–9 (31 × 8), with paired collecting ducts extending anteriorly; terminus indeterminate. Excretory pore terminal.

3.2.5 Taxonomic summary

Type-host: *Arothron nigropunctatus* (Bloch & Schneider), Black-spotted puffer (Tetraodontiformes: Tetraodontidae).

Type-locality: Flora Reef, northeast Queensland, Australia (17°11'S, 146°17' E).

Other locality: Off Lizard Island, northeast Queensland, Australia (14°38'S, 145°27'E).

Site in host: Within gill filaments. Trapped eggs found in gill lamellae.

Prevalence: Flora Reef- Two of three fish infected; one with 8 worms, the other with only eggs detected. Lizard Island- Two of five fish infected; one with 2 worms, the other with only eggs detected. None of three from the Moreton Bay region or seven from the Capricorn-Bunker group (Heron Island, Northwest Island and Wistari Reef) were infected.

Type-material: Holotype (QMXX) and 4 paratypes, including 1 hologenophore.

Representative DNA sequences: ITS2 rDNA, two identical replicates from Flora Reef and Lizard Island (one sequence submitted: GenBank XX); partial 28S rDNA, two identical replicates from Flora Reef and Lizard Island (one sequence submitted: GenBank XX).

Etymology: This species is named for Daniel C. Huston of The University of Queensland, for his enthusiastic support of the authors' work.

3.2.6 Remarks

Psettarium hustoni n. sp. is just the second species of *Psettarium* after *P. ogawai* reported with multiple testes. *Psettarium hustoni* n. sp. differs from *P. ogawai* in having entirely post-caecal testes, as opposed to partially lateral to the caeca, and by having the testes arranged in opposite pairs rather than offset relative to one another. The ovary of *P. hustoni* n. sp. is also consistently oblong, whereas that of *P. ogawai* is consistently spherical. Lastly, the posterior testis of *P. hustoni* n. sp. is consistently larger than that of *P. ogawai*, occupying almost the entirety of the post-uterine posterior extremity. *Psettarium hustoni* n. sp. differs from *P. ogawai* by 15 bp in the ITS2 rDNA region and 12 bp in the partial 28S rDNA region. It differs from all other species of tetraodontiform-infecting *Psettarium* for which molecular data are available by 11–40 bp in the partial 28S rDNA region and 7–19 bp in the ITS2 rDNA region. All specimens of *P. hustoni* n. sp. were found entirely within the afferent gill arteries of individual gill filaments, usually accompanied by accumulations of eggs trapped in the lamellae (Figs 4B, 5). No other species of *Psettarium* has been reported from within the gill filaments.

***3.2.7 Psettarium martini* n. sp.** **Fig. 1C, 2C, 4D**

Description: [Based on 27 whole-mounted specimens, including two hologenophores]. Body variably broad, lanceolate, ventrally concave, margins narrow slightly unequally at level of male genital pore, 1605–3295 × 231–614 (2539 × 370), 3.96–9.63 (7.0) times longer than broad. Tegumental spines minute, straight, 3 long, arranged in marginal transverse rows that wrap dorsoventrally, spaced 3 apart and with 8 spines per row throughout; rows begin 5–13 (11) from base of oral sucker, initially 6–9 (7) in length, increasing within first 10 rows to 8–21 (14) in length before decreasing towards posterior extremity to 6–11 (9) in length, with rows ending 7–30 (19) from margins of excretory pore. Nerve cords 7 in diameter, run length of body 42–101 (65) from body margins, well-defined. Dorsal nerve commissure 112–177 (151) from anterior extremity, 49–101 (67) across. Oral sucker well defined, 22–37 × 29–46 (28 × 39), with five concentric rings of fine spines. Mouth a simple pore, terminal to just ventrally subterminal. Oesophagus gently sinuous throughout, 599–991 (821), occupying 26.0–38.8% (32.7%) of total body length. Intestinal bifurcation medial in anterior half of body; caeca form X-shape. Anterior caeca straight with some lateral diverticulation, subequal in length. Left anterior caecum 139–239 (177); right anterior caecum 151–279 (187). Posterior caeca mostly straight, with some constrictions or diverticulation in some specimens, equal to greatly unequal in length. Left posterior caecum 531–1671 (1151); right posterior caecum 444–1635 (1190). Posterior caeca 3.3–11.0 (7.4) times longer than anterior caeca; total caecal length 42.0–61.6% (55.7%) of total body length.

Anterior testis not deeply lobed, margins irregularly undulate, inter-caecal, with lateral margins extending beyond bounds of posterior caeca and nerve cords, and posterior margins extending on both sides to almost envelop ovary laterally, 865–2233 × 124–418 (1609 × 235). Vas deferens originates medially from posterior margin of testis, passes over ovary and anterior portion of uterus, before expanding slightly and bifurcating posterior to cirrus-sac, with one duct leading to cirrus-sac, and other

duct passing dextrally dorsal to uterus and ventral to oviduct and vitelline duct, meeting posterior testis. Posterior testis highly variable in turgidity and definition, occupies much of space dextrolateral and posterior to rest of terminal genitalia, 30–91 (69) from posterior extremity, 17–157 × 14–193 (79 × 48). Cirrus-sac reniform, filled mainly by mass of glandular cells surrounding broad, poorly-delineated duct, 25–182 × 17–93 (80 × 42). Male genital pore dorso-sinistral, raised from body on dorsally-directed protuberance. Protuberance conical, 14–43 × 23–76 (29 × 42), laterally marginal or nearly so, 117–304 (185) from posterior extremity.

Ovary variable but generally sub-triangular, deeply lobed, sub-medial to dextral in body, almost completely enveloped by testis, 40–190 × 58–258 (101 × 151). Oviduct originates medially from posterior margin of ovary, passes posteriorly, then anteriorly to meet oötype. Oötype well-defined, medial or nearly so in body, 83–197 (117) from posterior extremity. Uterus tightly coiled, passing anteriorly nearly medially in body to posterior ovarian margin, then posteriorly to open at female genital pore. Female genital pore dorso-sinistral, anterior and dextral to cirrus-sac and male genital pore; 50–182 (76) from body margin, 158–424 (230) from posterior extremity. Eggs seen in gills and *in utero*, spheroidal, 58–66 × 38–47 (61 × 44). Vitelline follicles extensive, dense, evenly distributed from near anterior extremity to level of female genital pore, extending laterally beyond nerve cords almost to body margins. Vitelline duct runs medially, traceable from near caecal bifurcation to oötype, dorsal to rest of genitalia.

Excretory vesicle 15–43 × 5–13 (32 × 9), with paired collecting ducts extending anteriorly, traceable as far as terminal genitalia; terminus indeterminate. Excretory pore terminal.

3.2.8 Taxonomic summary

Type-host: *Arothron stellatus* (Bloch & Schneider), Starry puffer (Tetraodontiformes: Tetraodontidae).

Type-locality: Off Amity Point, North Stradbroke Island, Moreton Bay, southeast Queensland, Australia (27°23'S, 153°26'E).

Other localities: Moreton Bay region, southeast Queensland, Australia (27°23'S, 153°19'E).

Gold Coast Seaway, Gold Coast, southeast Queensland, Australia (27°56'S, 153°24'E).

Site in host: Gill arteries. Trapped eggs found in gill lamellae, rarely in heart.

Prevalence: Gold Coast- One out of one fish infected with one worm. Moreton Bay- Four of four fish infected; three with 1–60 worms and one with only fresh eggs in gills detected.

Type-material: Holotype (QMXX) and 28 paratypes, including 1 hologenophore (QMXX).

Representative DNA sequences: ITS2 rDNA, two identical replicates (one sequence submitted: GenBank XX); partial 28S rDNA, two identical replicates (one sequence submitted: GenBank XX).

Etymology: This species is named for Storm B. Martin of The University of the Queensland, for his help in obtaining the majority of hosts for this species and support of the authors' work.

3.2.9 Remarks

Psettarium martini n. sp. possesses a single large anterior testis that extends anteriorly beyond the level of the caecal bifurcation, but not anterior to the anterior caeca. In that respect, it resembles *P. japonicum*, *P. rachycentri*, *P. sinense* and *P. tropicum*. It differs from all four species in possessing a posterior testis. *Psettarium martini* n. sp. also differs from *P. rachycentri* in having an ovary that is not deeply lobed and is dextral (not medial) in the body, and in not having posterior caeca that have dendritic lateral projections and that extend to the level of the ovary. It differs from *P. sinense* in not having a testis with deeply-lobed lateral margins, and from *P. tropicum* in the relative straightness of the posterior caeca, the tegumental spines not fusing to become single blades, and an unlobed (rather than bilobed) ovary. *Psettarium martini* n. sp. most closely resembles *P. japonicum*, but is smaller (1605–3295 µm *versus* 5350–6300 µm), has a longer oesophagus relative to total body length (average of one-third total body length as opposed to one-sixth), has a female genital pore posterior to rather than level with the ovary, an oötype that is not posterior to the rest of the uterine coils, and a uterus that is more tightly and regularly coiled. Molecular data are not available for *P. japonicum*; of those species for which molecular data are available, *P. martini* n. sp. is most similar to *P. hustoni* n. sp., differing by 2 bp in the ITS2 rDNA region and by 3 bp in the 28S rDNA region, and from all other tetraodontiform-infecting species of *Psettarium* by 4–9 bp in the ITS2 rDNA region and 11–22 bp in the 28S rDNA region.

***3.2.10 Psettarium yoshidai* n. sp.** **Fig. 1D, 2D, 4E**

Description: [Based on 3 whole-mounted specimens]. Body broadly lanceolate, ventrally concave, margins narrow slightly unequally at level of male genital pore, 1296–2853 × 188–361 (2290 × 264), 6.9–11.2 (8.7) times longer than broad. Tegumental spines minute, straight, less than 2 long, arranged in marginal transverse rows that wrap dorsoventrally; rows spaced 2 apart throughout, begin 8–11 (10) from base of oral sucker, initially 6–7 (7) in length and bearing 3–4 (4) spines each, increasing within first 10 rows to 7–12 (10) in length, bearing 6–8 (7) spines each, before decreasing towards posterior extremity to 6 in length, bearing 4–5 spines each, with rows ending 14–22 (18) from posterior extremity. Nerve cords 7 in diameter, run length of body 25–55 (38) from body margins, well-defined. Dorsal nerve commissure 96–140 (125) from anterior extremity, 44–74 (56) across. Oral sucker well defined, aspinous, 17–30 × 25–49 (25 × 40). Mouth a simple pore, terminal to just ventrally subterminal. Oesophagus gently sinuous throughout, 490–753 (603), occupying 19.9–37.8% (28.5%) of total body length. Intestinal bifurcation medial in anterior half of body; caeca form X-shape. Anterior caeca straight with minimal diverticulation, subequal in length. Left anterior caecum 121–177 (156); right anterior caecum 118–203 (157). Posterior caeca straight, greatly unequal in length. Left posterior caecum 409–1131 (793); right posterior caecum 318–1104 (765). Posterior caeca 3.5–6.4 (5.2) × longer than anterior caeca; total caecal length occupying 38.4–46.8% (42.0%) of total body length.

Anterior testis single, not deeply lobed, margins irregular, inter-caecal, with margins extending laterally beyond bounds of posterior caeca and nerve cords, posteriorly beyond extent of posterior caeca, 736–1129 × 206–246 (933 × 226). Vas deferens originates medially from posterior margin of testis, passes over ovary and anterior portion of uterus, before expanding slightly and bifurcating dorsal to posterior margin of cirrus-sac, with one duct leading to cirrus-sac, and other duct passing dextrally dorsal to uterus and ventral to oviduct and vitelline duct, meeting posterior testis. Posterior testis lobed, well-developed, occupying most of post-uterine space, posterior and partially lateral to rest of genitalia, 270–

359 × 109–158 (315 × 134). Cirrus-sac reniform, filled mainly by mass of glandular cells surrounding broad, poorly-delineated duct, 58–105 × 33–51 (79 × 43). Male genital pore dorso-sinistral, somewhat raised from body on indistinct dorsally-directed protuberance, opening 23–50 (33) from lateral body margin, 173–457 (338) from posterior extremity.

Ovary oblong, lobed, medial, immediately posterior to testis, 93–134 × 153–200 (114 × 177). Oviduct originates medially from posterior margin of ovary, passes posteriorly, then anteriorly to meet oötype. Oötype well-defined, medial or nearly so in body, 107–364 (249) from posterior extremity. Uterus coiled, passing anteriorly to posterior ovarian margin, then posteriorly to open at female genital pore. Female genital pore dorso-sinistral, anterior and dextral to cirrus-sac and male genital pore; 31–70 (51) from body margin, 209–542 (395) from posterior extremity. Eggs seen in gills and *in utero*; spheroidal, 42–44 × 28–32 (43 × 30). Vitelline follicles extensive, dense, evenly distributed from near anterior extremity to level of female genital pore, extending laterally beyond nerve cords almost to body margins. Vitelline duct runs medially, traceable from near caecal bifurcation to oötype, dorsal to rest of genitalia.

Excretory vesicle observed in one specimen 20 × 5, with paired collecting ducts extending anteriorly, traceable as far as terminal genitalia; terminus indeterminate. Excretory pore terminal.

3.2.11 Taxonomic summary

Type-host: *Arothron mappa* (Lesson), Map puffer (Tetraodontiformes: Tetraodontidae).

Type-locality: Gold Coast Seaway, Gold Coast, southeast Queensland, Australia (27°56'S, 153°24'E).

Site in host: Gill vessels.

Prevalence: Gold Coast- One of one fish infected with eight worms. Lizard Island - One fish not infected.

Type-material: Holotype (QM XX) and 4 paratypes, including 1 hologenophore (QM XX).

Representative DNA sequences: ITS2 rDNA, two identical replicates (one sequence submitted: GenBank XX); partial 28S rDNA, two identical replicates (one sequence submitted: GenBank XX).

Etymology: This species is named for Dr Teruaki Yoshida of Universiti Malaya Sabah, Malaysia, for his continuing support of the first author from an early age, providing encouragement and advice towards pursuing a career in the field of marine science.

3.2.12 Remarks

Among species of *Psettarium*, *P. yoshidai* n. sp. most closely resembles *P. jimbaranense*, described from the gills of *Arothron reticularis* from off Bali, Indonesia, in possessing a narrowly lanceolate body, a large testis extending laterally beyond the margins of the posterior caeca, and a relatively large and well-developed posterior testis. Morphologically, *P. yoshidai* n. sp. can be distinguished from *P. jimbaranense* by the unlobed, smoothly ovoid ovary located medially in the body, in contrast to the irregularly oblong, lobed ovary of *P. jimbaranense*, located dextrally in the body. The two species also differ by 5 bp in the ITS2 rDNA region and 15 bp in the partial 28S rDNA region. *Psettarium yoshidai* n. sp. differs from all

other tetraodontiform-infecting species of *Psettarium* for which sequence data are known by 3–8 bp in the ITS2 rDNA region and 7–20 bp in the 28S rDNA region.

3.2.13 Psettarium nolani (Bray, Cribb & Littlewood, 2013) Yong, Cutmore, Bray, Miller, Semarariana, Palm & Cribb, 2016 Fig. 4A, 6

Type-host: *Arothron meleagris* (Anonymous), Guineafowl puffer (Tetraodontiformes: Tetraodontidae).

Type-locality: Moorea, French Polynesia, east Pacific.

New localities: Moreton Bay region, southeast Queensland, Australia (27°23'S, 153°19'E).

Gold Coast Seaway, Gold Coast, southeast Queensland, Australia (27°56'S, 153°24'E).

Mackay Marina, Mackay, north Queensland, Australia (21°06'S, 149°13'E).

Lizard Island, north Queensland, Australia (14°41'S, 145°27'E).

New hosts: *Arothron hispidus* (Linnaeus), Stars-and-stripes puffer (Gold Coast, Moreton Bay, Mackay, Lizard Island).

Arothron manilensis (de Procé), Narrow-lined puffer (Gold Coast, Mackay).

Arothron stellatus (Bloch & Schneider), Starry puffer (Moreton Bay).

Site in host: Primarily in mesenteric vessels and aorta, with occasional individuals found in branchial vessels. Trapped eggs found in heart, intestinal wall, liver, spleen and kidney.

Prevalence: *Arothron hispidus*: Gold Coast – Five of six fish infected; four with 1–26 worms, one with only eggs in gills detected. Lizard Island – Two of five fish infected with one worm each. Mackay – One fish not infected. Moreton Bay – Eight of 13 fish infected; two with 5–12 worms, six with only eggs in gills or heart detected.

Arothron manilensis: Gold Coast – One of three fish infected. Mackay – Two of five fish infected. No infections detected in two fish from Lizard Island or 12 from Moreton Bay.

Arothron stellatus: Moreton Bay – One of four fish infected with two worms.

New material: Voucher specimens (QM XX) including 1 hologenophore (QM XX).

Representative DNA sequences: ITS2 rDNA, two identical replicates ex *A. hispidus* from Moreton Bay and Lizard Island (GenBank XX) and one from Mackay ex *A. manilensis* (GenBank XX); partial 28S rDNA, two identical replicates from Gold Coast (ex *A. hispidus*) and Mackay (ex *A. manilensis*) (one sequence submitted: GenBank XX).

3.2.14 Remarks

Molecular analysis showed that specimens of *P. nolani* from Mackay (ex *A. manilensis*) were identical in both ITS2 and partial 28S rDNA regions to specimens of *P. nolani* from French Polynesia, while

specimens from southeast Queensland (ex *A*. *hispidus* from Moreton Bay and Gold Coast) and Lizard Island (ex *A*. *hispidus*) differed from the former by 1 bp in the ITS2 rDNA region. No molecular data exist for specimens of *P*. *nolani* found in *A*. *stellatus*. Australian specimens show little morphological difference relative to specimens from French Polynesia (Fig. 6). Some variation was observed in the dimensions of the anterior testis relative to total body length, its anterior extent not meeting the intestinal bifurcation and the posterior margin contacting the anterior margin of the ovary; all such variations, however, are still consistent with the concept of the species. *Arothron hispidus*, *A*. *manilensis* and *A*. *stellatus* represent new hosts for this species.

3.2.15 Psettarium pulchellum Yong, Cutmore, Bray, Miller, Semarariana, Palm & Cribb, 2016 Fig. 4C, 7

Type-host: *Arothron manilensis* (de Procé), Narrow-lined puffer (Tetraodontiformes: Tetraodontidae).

Other host: *Tylerius spinosissimus* Regan, Spiny blaasop (Tetraodontiformes: Tetraodontidae).

Type-locality: Kedonganan Beach, Bali, Indonesia.

New localities: Off Peel Island, Moreton Bay, southeast Queensland, Australia (27°30'S, 153°21'E).

Mackay Marina, Mackay, north Queensland, Australia (21°06'S, 149°13'E).

Lizard Island, north Queensland, Australia (14°41'S, 145°27'E).

Site in host: In afferent arteries, within gill arches.

Prevalence: Lizard Island – One of two fish infected with a single worm. Mackay – One of five fish infected with four worms. Moreton Bay – Six of 12 fish infected with 1–24 worms. Three fish from Gold Coast not infected.

New material: Voucher specimens (QMXX) including 1 hologenophore (QMXX). All specimens ex *A*. *manilensis*.

Representative DNA sequences: ITS2 rDNA, two identical replicates from Mackay and Moreton Bay (GenBank XX); partial 28S rDNA, two identical replicates (one sequence submitted: GenBank XX).

3.2.16 Remarks

The new specimens of *P*. *pulchellum* were morphologically indistinguishable from those originally described from fish collected off Bali, Indonesia by Yong *et al.* [8] (Fig. 7). Specimens from two localities on the east QLD coast (Moreton Bay and Mackay) were identical in the ITS2 rDNA region, but differ from *P*. *pulchellum* infecting Balinese *Tylerius spinosissimus* by 1 bp and from Balinese *Arothron manilensis* by 2 bp; worms from east QLD and Bali were identical in the partial 28S rDNA region. These differences are interpreted as intraspecific variation. No molecular data were available for *P*. *pulchellum* from Lizard Island, as only a single specimen was collected. We interpret all these specimens as being conspecific with *P*. *pulchellum*.

3.3 Infection biology

Worms and eggs of five species (*P. hustoni* n. sp., *P. martini* n. sp., *P. pandora* n. sp., *P. pulchellum* and *P. yoshidai* n. sp.) were found in the gills of infected fish. Almost all eggs seen in gills were evidently viable and in varying states of embryonation. Most were individually scattered in lamellar tissue, but some formed accumulations enveloped by a host immune response (e.g. Fig. 4D). Eggs of two mesentery-infecting species, *P. hawaiiense* n. comb. and *P. nolani*, were observed on occasion in the gills (Fig. 8F), in visceral organs, particularly the liver (Fig. 4A, 8C), but also in the spleen (Fig. 8D), gallbladder (Fig. 8E) and kidney. Live eggs were observed in the faeces of one fish (Fig. 8G), and live miracidia (species indeterminate) seen in the water in which individual *A. hispidus* were held (Fig. 8H). For both species, cysts formed by agglomerations of dead and live eggs and, on one occasion, degenerate worms (not pictured), were also noted in the mesenteries and on the surface of mesenteric vessels (Fig. 8A, B).

Heavy accumulations of aporocotylid eggs were seen in intestinal sections of all three studied fishes. Eggs were primarily observed in the mucosal layer of the intestine, either as individuals or aggregates (Fig. 9A). In some cases, eggs reached as far as the villi and even abutted the margin of the lamina propria (Fig. 9B, C), with some evidence of pathological degradation of the laminar epithelium (Fig. 9C). Only on one occasion was evidence seen of erosion of the laminar epithelium by an egg and associated pathology (Fig. 9D). Eggs embedded in the mucosa were usually surrounded by an acute inflammatory and granulomatous response; the sections were not tested for calcification response. Some eggs appeared to be dead and degraded; other eggs appeared viable. Host inflammatory response was observed in the form of accumulations of immune cells; these cells were not characterised. On some occasions, a granulomatous response was observed, characterised by monocytic infiltration and degradation of the eggs.

3.4 Molecular results

3.4.1 Overview

Alignment of the ITS2 rDNA dataset was limited to comparison of the species reported in this paper and those of other tetraodontid-infecting aporocotylids for which sequence data were available. The alignment yielded 459 characters for analysis. Bayesian inference and Maximum Likelihood analyses of the ITS2 dataset resulted in near-identical phylograms, though that of the Maximum Likelihood analysis generally showed lower nodal support than the Bayesian analysis (Fig. 10). The ITS2 rDNA analysis showed that all species encountered in this study formed a clade with other tetraodontid-infecting species of *Psettarium*. ITS2 rDNA sequences of *P. nolani* from Mackay were identical to those generated by Yong *et al.* [8] for individuals from French Polynesia, and both were 1 bp different from specimens from Gold Coast and Lizard Island (Table 3). The variation was a T-C substitution at base #265 in our alignment. Individuals of *P. pulchellum* from Moreton Bay and Mackay were identical in the ITS2 rDNA region, and were 1 bp different from an individual that infected *Tylerius spinosissimus* and 2 bp different from one that infected *Arothron manilensis*, both also from Bali (Table 3). These variations were a G-A substitution at base #188 and a C-T substitution at base #333; the latter difference was only observed in the specimen infecting *A. manilensis* from Bali. No differences in the ITS2 rDNA region were detected between individuals of *P. hustoni* n. sp. from Flora Reef and Lizard Island, or *P. hawaiiense* n. comb. from Moreton Bay and Mackay.

Partial 28S rDNA data were generated for the four new species and for specimens identified as *P. nolani* and *P. pulchellum* from Moreton Bay and *Psettarium hawaiiense* n. comb. from Mackay and Moreton Bay. Alignment of the partial 28S rDNA dataset for a total of 53 putative aporocotylid species, yielded 1196 characters for analysis. Bayesian inference and Maximum Likelihood analyses produced phylograms with near-identical topologies (Fig. 11). In both analyses, all tetraodontiform-infecting taxa, except for the monacanthid-infecting threadlike species *Ankistromeces mariae* Nolan & Cribb, 2004, formed a strongly-supported clade to the exclusion of all other aporocotylids. There was some discrepancy in the inter-relationships between its member taxa in the two analyses, but these were characterised by low nodal support. This clade was sister to species of threadlike aporocotylids from the genera *Ankistromeces* Nolan & Cribb, 2004 and *Phthinomita* Nolan & Cribb, 2006, as well as *Skoulekia meningialis* Alama-Bermejo, Montero, Raga & Holzer, 2011 from the Mediterranean sparid *Diplodus vulgaris* Geoffroy Saint-Hilaire. The phylogenetic tree presented by the 28S analysis did not correlate with any patterns of host range, ecology, or infection site within the tetraodontid hosts.

In our initial analyses, sequences of *Psettarium anthicum* and a closely-related, undescribed taxon from Nha Trang, Vietnam, referred to as *P.* cf. *anthicum* [31], were included for the first time in a phylogenetic analysis of the family. These sequence data, generated from specimens collected from cobia (*Rachycentron canadum* Linnaeus) from the Gulf of Mexico and Vietnam respectively [31], formed a clade phylogenetically distant from all other species of *Psettarium*. These taxa instead formed a clade sister to that formed by species of *Paradeontacylix* McIntosh, 1934, and *Cardicola* Short, 1953. Bayesian inference and Maximum Likelihood analyses of both partial 28S and ITS2 rDNA regions showed identical topologies in this regard (the latter not shown). It is clear that *P. anthicum* does not belong in the genus *Psettarium* and should be recognised in its own genus. No other existing genus is available and we therefore propose the new genus *Cardallagium* n. gen. for this species. *Psettarium anthicum* is reclassified as *Cardallagium anthicum* n. comb and *P.* cf. *anthicum* is transferred to this genus. *Cardallagium* n. gen. is diagnosed below.

3.4.2 Cardallagium n. gen.

Diagnosis: Body extremely elongate, broadest at level of testis, with distinct lateral hooked bend at posterior end and sinistral posterolateral protuberance projecting laterally from body margin. Tegument rugose. Tegumental spines in lateral transverse rows, either running along entire length of body margins or ending 26–36% of total body length from posterior extremity. Oral sucker present or possibly absent, unspined. Mouth medioventrally subterminal. Oesophagus mostly straight, with up to three slight curves. Caeca form H-shape. Posterior caeca with distinct lateral projections, sometimes thornlike in appearance, 19–43 times longer than anterior caeca, occupying up to 52% of total body length. Testis single, medial, entirely post-caecal, with margins distinctly lobed. Cirrus-sac present, contains seminal vesicle, ejaculatory duct and cirrus. Posterior testis or auxiliary seminal vesicle absent. Male genital pore at extremity of sinistral posterolateral protuberance. Ovary post-testicular, medial, distinctly lobed. Oötype posterior to rest of genitalia. Uterus convoluted, with dense coils primarily occupying space between ovary and cirrus-sac. Female genital pore dorsal, submedial, anterior and dextral to sinistral posterolateral protuberance. Eggs spheroid, thin-shelled. Vitellarium comprising extensive network of narrow interconnecting branching bands, extends from level of ventrolateral nerve cord to anterior margin of ovary, ventral to caeca and testis. Known only from *Rachycentron canadum* (Rachycentridae).

In endocardial wall of heart, with bodies laced through spongy tissue and attached *via* midsection with anterior and posterior portions free in cardiac lumen.

Type-species: *Cardallagium anthicum* (Bullard & Overstreet, 2006) n. comb.

Other species: *Cardallagium* cf. *anthicum* per Warren et al. (2017)

Etymology: The name *Cardallagium* is derived from the Greek words "*kardia*" (heart) and "*allagi*" (change), in reference to the pathological deformities caused by attachment of this parasite to the inner walls of its host's heart, as described by Bullard & Overstreet [3] and Warren *et al.* [31].

Differential diagnosis: In having a combination of a narrowly elongate body with distinct hooked posterior bend, X-shaped caeca, a single testis, cirrus-sac, posterolateral protuberance at male genital pore and post-ovarian uterine coils, species of *Cardallagium* n. gen. can be differentiated from those of all other blood flukes except *Cardicola* and *Psettarium*. They differ from *Cardicola* species by having an entirely post-caecal testis, a hooked posterior bend to the body and the oötype being posterior to the rest of the terminal genitalia or nearly so (in most species of *Cardicola*, the oötype is lateral or anterior to uterine coils, or is level with or anterior to the cirrus sac and male genital pore). Species of *Cardallagium* n. gen. are most similar to species of *Psettarium*. They differ in much greater total body length (9,500–11,000 µm for *C. anthicum* n. comb., versus 1300–6300 µm for species of *Psettarium*), as well as having a single post-caecal testis (the only species of *Psettarium* with post-caecal testes have multiple testes), no posterior testis (not found in all species of *Psettarium*), a more pronounced posterolateral protuberance at the male genital pore and more convoluted uterine coils. The infection habit of *Cardallagium* spp. is unique among heart-infecting aporocotylids. Apart from the threadlike species of *Ankistromeces* and *Phthinomita*, no other aporocotylids infect the endocardial walls and thread their bodies into the spongy tissue, generally living free in the cardiac lumen instead. The infection habit of species of *Cardallagium* n. comb., whereby they loop their bodies into the cardiac walls, leaving the posterior and anterior ends free in the lumen, is apparently unique among aporocotylids. An oral sucker was not described for *C. anthicum* n. comb., but was noted as present in *C.* cf. *anthicum* n. comb. by Warren et al. (2017). We suspect that the feature was missed in the original description of *C. anthicum* n. comb., as may have occurred for many other species of aporocotylids.

4. Discussion

4.1 Implications for systematics, phylogeny and host range

The descriptions of four new species, the addition of *Paracardicola hawaiensis* (now *Psettarium hawaiiense* n. comb.) and the removal of *Psettarium anthicum* mean that the genus *Psettarium* now includes 14 species. All but two of these infect tetraodontiform fishes; *P. pseudupenei* is known only from goatfish (Mullidae) and *P. rachycentri* is known only from the cobia (Rachycentridae) [3, 4]. Of the remaining 12 species, one infects an ostraciid and the other 11 infect tetraodontids. Only three species of tetraodontiform-infecting aporocotylids are now recognised as being from genera other than *Psettarium*: *Paradeontacylix odhneri*, which infects the tetraodontid *Takifugu porphyreus* (Temminck & Schlegel), *Rhaphidotrema kiatkiongi*, which infects the stars-and-stripes puffer, *A. hispidus*, and the

threadlike *Ankistromeces mariae*, which infects the monacanthid *Meuschenia freycineti* (Quoy & Gaimard) [6, 32, 33]. The molecular phylogenetic affinities of *R. kiatkiongi* remain unknown. This species was described by Yong & Cribb [6] from Lizard Island on the far northern GBR, and is remarkably different from all known aporocotylids in the morphology of its terminal genitalia, particularly in the possession of a sclerotised penis stylet. *Paradeontacylix odhneri* also remains enigmatic; it is known from a single specimen, now lost, collected from Peter the Great Bay in far-eastern Russia, and originally described as *Aporocotyle odhneri* [32]. McIntosh [34], in erecting the new genus *Paradeontacylix*, transferred it to that genus. Given that all other species of *Paradeontacylix* infect very different hosts (perciform fishes of the family Carangidae), we suspect that this genus placement is erroneous and that *P. odhneri* will ultimately prove to be a species of *Psettarium*. It is notable that the sole specimen of *P. odhneri* had multiple testes, and this feature is now known from two species of *Psettarium*. We do not, however, propose to re-assign this species, due to the brevity of the original description and lack of access to the specimen.

It is noteworthy that species belonging to groups sister to *Psettarium* show relatively constrained host ranges. Species of *Skoulekia* are, so far, limited to fishes from the perciform family Sparidae [35, 36]. Four putative species of *Ankistromeces* for which sequence data are available form a strongly-supported clade of closely-related species, infecting species from two orders of fishes, chiefly in the rabbitfish family (Perciformes: Siganidae). The affinities of *A. mariae* with other threadlike aporocotylids are not in doubt, having been demonstrated by both morphological and molecular analysis [37, 38]. The infection of a tetraodontiform fish by an aporocotylid species not closely-related to species of *Psettarium* does not fit with the notion of constrained host range. It may be that this represents an isolated host-switching event, with monacanthids being otherwise poor hosts for blood flukes. Alternatively, it may indicate that there are more monacanthid-infecting aporocotylid species to be discovered.

Psettarium pandora n. sp. is the first aporocotylid described from a boxfish (family Ostraciidae). Our sampling of this family is too limited to establish either the geographical distribution of this species or whether the 25 recognised ostraciid species harbour further aporocotylid richness. The three other new species described here all infect species of pufferfishes from the genus *Arothron*. Their discovery brings the number of known pufferfish-infecting aporocotylid species to 13, with the largest known radiation of these species occurring among *Arothron*-infecting species. So far, seven of the 16 known species of *Arothron* are hosts of aporocotylids, being infected by nine species. *Arothron hispidus* has the highest-known aporocotylid richness of all tetraodontids; in addition to being infected by *P. hawaiiense* and *P. nolani*, it is also infected by *Rhaphidotrema kiatkiongi* [6]. Three other *Arothron* species (*A. manilensis*, *A. reticularis* and *A. stellatus*) are infected by two aporocotylid species and the remaining three (*A. mappa*, *A. nigropunctatus* and *A. stellatus*) by one species each. It has been postulated that some lineages of tetraodontid fishes (e.g. *Takifugu*) have undergone, or are undergoing, explosive speciation [39]. The *Arothron* lineage also appears to have undergone relatively recent and rapid diversification in the post-Miocene era [40] and the genus *Psettarium* appears to have undergone comparably rapid diversification with it. We predict that most, if not all, species of *Arothron* will ultimately prove to be host to species of *Psettarium*.

Yong *et al.* [8] reported dramatic variation in the testes of three *Arothron*-infecting aporocotylid species from Bali, Indonesia. However, they found close molecular relationships between the taxa and recognised all three as belonging to *Psettarium*. Our new findings support this interpretation, as the nine species for which sequences are available form a strongly-supported clade, comparable to those formed

by species of *Paradeontacylix* and *Aporocotyle*. As observed by Yong *et al.* [41], the species of *Cardicola* show far more molecular diversification than the genera discussed above and it is possible that this genus requires subdivision. The form of the testes in species of *Psettarium* is exceptionally variable. The anterior testis may be small and compact, massive or divided into distinct multiple testes. The posterior testis can be smaller than the anterior testis but apparently functional (e.g. in *P. hawaiiense* n. comb., *P. hustoni* n. sp. and *P. yoshidai* n. sp.), present but apparently non-functional (e.g. *P. martini* n. sp., *P. ogawai* and *P. pulchellum*), or completely absent (*P. japonicum*, *P. pandora* n. sp., *P. sinense* and *P. tropicum*). A notable inference from our new phylogenetic analysis is that multiple anterior testes have evolved more than once within the clade of *Psettarium*; *P. hustoni* n. sp. and *P. ogawai* are not each other's closest relatives. Similarly, *P. pandora* n. sp. and *P. sinense* both lack a posterior testis completely but are not closely related to one another. These findings suggest that the form of the testes has been exceptionally plastic in the evolution of the species of this genus. Whether the lack or presence of a posterior testis is the ancestral state among species of *Psettarium*, with the feature gained or lost several times in the evolutionary history of this group, remains unclear. It will be of great interest to sample other, more basal families of tetraodontiform fishes, such as spikefishes (Triacanthodidae) and three-toothed puffers (Triodontidae), to assess any aporocotylids that may be present.

The closeness in relationship between most tetraodontiform-infecting species of aporocotylids is particularly interesting due to the restriction of species of the genus *Psettarium* to tetraodontiform fishes. Of the eleven putative species recognised within the genus *Psettarium* prior to this study, seven infect tetraodontid fishes and five were demonstrated to have a very close molecular phylogenetic relationship, with as few as 8–10 base-pair differences between species despite high morphological disparity [8]. Bullard & Overstreet [3], in describing the rachycentrid-infecting *P. anthicum* (now *Cardallagium anthicum* n. comb.), also re-assigned species of the genus *Psettarioides* Lebedev & Parukhin, 1972, which included a further rachycentrid-infecting species (*P. rachycentri*) and one from a mullid (*P. pseudupenei*), to the genus *Psettarium*. The fact that *C. anthicum* n. comb. (and the highly closely-related *C.* cf. *anthicum* n. comb., also from a rachycentrid) has been demonstrated to be phylogenetically distantly-related to species of *Psettarium*, along with the tightness of the clade formed by tetraodontid-infecting species, casts suspicion upon the affinities of *P. pseudupenei* and *P. rachycentri*. Sequence data for the type-species of this genus, *P. japonicum*, as well as of *P. pseudupenei* and *P. rachycentri*, are thus highly desirable. We predict that, ultimately, the concept of *Psettarium* will be limited to tetraodontiform-infecting taxa.

4.2 Infection biology of Arothron*-infecting* Psettarium *species*

Both gill and mesenteric vessels can be regarded as primary infection sites for species of *Psettarium*, with three species (*P. jimbaranense*, *P. ogawai* and *P. pulchellum*) previously noted from the former and four (*P. hawaiiense* n. comb., *P. japonicum*, *P. nolani*, *P. sinense* and *P. tropicum*) from the latter. Most of the species of *Psettarium* encountered in this study (*P. martini* n. sp., *P. pandora* n. sp. and *P. yoshidai* n. sp.) were found in the branchial arteries of the gills, and one species, *P. hustoni* n. sp., was only found within the gill filaments. Some eggs and worms of *P. hawaiiense* n. comb. and *P. nolani* were found in the branchial vessels, but those species were overwhelmingly encountered in the mesenteric vessels and aorta.

In many instances, the detection of worms was preceded by the discovery of eggs trapped within the tissues of infected organs. Eggs of all gill-infecting species were encountered trapped within lamellar tissue of gill filaments of infected hosts, with eggs in varying stages of degradation also encountered in the heart. Eggs of mesentery-infecting species were encountered in both the mesenteries themselves and organs such as the liver, kidney and spleen. In the heaviest infections, tens of thousands of eggs were observed accumulated in affected organs. Eggs in the mesenteric tissues and vessels formed cysts similar to those reported by Bray *et al.* [7] for *P. nolani*. These cysts were formed in association with multiple eggs and, on one occasion, degenerate worms, appearing consistent with venal thickening and phlebitic reaction, as described by Wood & Bacha [14] and Hurst *et al.* [42] for schistosomatid infections.

Histopathological analysis of the intestines of *A. hispidus* heavily infected by both *P. hawaiiense* n. comb. and *P. nolani* showed heavy accumulations of eggs in the intestinal lining. While sectioning precluded identification of the eggs to species, it is presumed that those of both *P. hawaiiense* n. comb. and *P. nolani* were represented. The egg accumulations were concentrated in the mucosal layer of the intestine. Many dead and degraded eggs were observed adjacent to apparently-viable eggs in the same accumulations. Liu *et al.* [9] noted the presence of "empty eggshells" in the kidney of *Takifugu rubripes* infected by *Psettarium sinense*, and speculated that the miracidia hatched while trapped in the kidney and were excreted *via* the urine. Most of the "empty" eggs we encountered in the kidney and other organs appeared degraded by host pathological reaction rather than from hatching, being often tanned and surrounded by fibrocytic response, so we do not think that miracidia hatching while trapped in host organs is likely. Rather, our findings indicate that the egg biology of mesentery-infecting aporocotylids is similar to that of schistosomatids, in which eggs shed passively into the bloodstream leave the host through the faeces or urine, having passed through the gut or bladder wall, and only hatch once they encounter the external environment [14, 43].

It is thought that schistosomatid eggs are either laid by the worms into the intestinal lining through direct penetration, or are carried into the intestinal lining (or other organs) *via* passive circulation. The eggshells of schistosomatids have complex interactions with host tissues which facilitate excretion of the eggs by infected organs [reviewed in-depth by 44]. These interactions are mediated by a range of protein secretions, including antigenic glycoproteins which induce granuloma formation [45], proteolytic enzymes [46, 47] and other proteins which function as virulence factors [48]. It is hypothesised that these proteins facilitate direct passage through the tissues or instigate inflammatory reactions that ultimately result in erosion of the gut wall, allowing the eggs to enter the lumen and escape with the faeces [49]. It may be that the eggs of aporocotylids use the same mechanisms to facilitate their escape. Hurst *et al.* [42] noted that schistosomatids [specifically *S. japonicum* (Katsurada, 1904)] cause massive intestinal lesions by laying large aggregations of eggs, which may ultimately rupture into the intestinal lumen. While we did not encounter lesions on such a scale, we did note instances of isolated lesions, such as would allow one or a small number of eggs to pass into the intestinal lumen. Only once was direct evidence of eggs penetrating the laminar epithelium and entering the gut lumen observed. The high attrition of aporocotylid eggs caused by host immune response means such events are clearly rare relative to the number of eggs actually laid. It has been observed (in cases of schistosomatid infections) that, should a host survive initial infection, pathological response to egg infection gradually improves and becomes more efficient, to the point of self-curing [42, 50]. It therefore may be the case that, over time, successful egg passage becomes rarer as host responses become more efficient at destroying the eggs and worms.

4.3 Biogeography of Arothron*-infecting aporocotylids*

The discoveries of *Psettarium hawaiiense* n. comb., *P. nolani* and *P. pulchellum* off eastern Australia represent significant range extensions for these species. The former was described by Martin [5] from Hawaii, over 7,600 km from eastern Australia. Bray *et al.* [7] described *P. nolani* from off Moorea, French Polynesia, over 5,800 km distant from eastern Australia, while *P. pulchellum* was originally recorded by Yong *et al.* [8] from Bali, Indonesia, over 3,200 km from Lizard Island. Interestingly, the host species for both *P. hawaiiense* n. comb. and *P. pulchellum* were the same across all localities, but *P. nolani* has exploited three host species (*Arothron hispidus*, *A. manilensis* and *A. stellatus*) different to the type-host (*A. meleagris*). *Arothron meleagris* is the most common *Arothron* species in the east Pacific, but is rare along coastal eastern Australia; indeed, we have never encountered this species anywhere in Australian waters despite over 20 years' collecting efforts. A high rate of speciation has been demonstrated among aporocotylids, both allopatrically in a wide-ranging host fish [e.g. species of *Paradeontacylix* McIntosh, 1934, in amberjack, *Seriola dumerili* Risso, as per Repullés-Albelda *et al.* [51] and Ogawa & Egusa [52]] and multiple cryptic species sympatrically infecting a single host species [e.g. species of *Ankistromeces* infecting mottled spinefoot, *Siganus fuscescens* (Houttuyn) (Perciformes: Siganidae), as noted by Nolan & Cribb [38] and Brooks *et al.* [37]]. In that light, it is interesting that these species of *Psettarium* show no evidence of speciation across such a wide range and different hosts. It is possible that analysis of different gene regions will reveal that these do indeed form complexes of cryptic species but, on the basis of our data, it appears they are genuinely widespread species.

The few examples of aporocotylids known to be truly circumglobally distributed, certain species of *Cardicola*, infect highly-vagile pelagic fishes–bluefin tunas (Scombridae: *Thunnus* spp.) [53]. The dispersive potential of species of *Arothron* is speculated to be high, given their putatively long pelagic larval durations (PLD), though PLD data exist for just one species of *Arothron*, *A. hispidus* [54] and, in any case, it seems unlikely that infections are transported by pelagic larval pufferfishes *in pelagia* rather than by the adult fishes. Indeed, there is evidence that the larvae of *Arothron* spp. may not actually disperse widely during the planktonic stage despite their long PLD [55]. Some *Arothron* puffers are regarded as sedentary, even territorial, though others, like *A. manilensis* and the pelagic *A. firmamentum* (Temminck & Schlegel), are known to be wide-ranging [56, 57]. These species may have been critical in enabling species of *Psettarium* to disperse so widely, given that aporocotylid miracidia and cercariae are regarded as short-lived, poor swimmers, with a limited capacity to survive *in pelagia* [58].

Our understanding of ocean current dynamics also makes the distribution patterns of these parasites interesting. Species of *Arothron* are postulated to have radiated between the late Miocene (8 mya) and early Pleistocene (2.5 mya) [59], with the Indo-West Pacific (IWP) region being the center of diversity for the genus. This postulation is supported by the fact that the numbers of species of *Arothron* decrease eastwards across the IWP, from nine in Indonesia and eight off South Africa, to just three in French Polynesia [60]; the speed of this radiation is indicated by the short branch lengths seen in molecular phylogenetic analyses of both species of *Arothron* and those of *Psettarium* [8, 59; this study]. The strong south-westerly current flowing east of Bali, Indonesia, roughly corresponding to the Wallace-Lydekker Line and a formidable marine biogeographical barrier [61, 62], has apparently neither hindered species like *P. pulchellum* from reaching both Bali and eastern Australia, nor resulted in wider speciation. This

may indicate that the prevailing currents, as noted by Carpenter & Springer [62], have had a stronger influence on the radiation and dispersal of species of *Psettarium* and *Arothron* across the tropical Indo-west Pacific region. For now, how certain species of *Psettarium* have become so widespread in the IWP region remains unknown. Comprehensive assessment of *Arothron* species across the tropical IWP will be imperative for understanding the true extent of aporocotylid richness and host range in this genus of fishes, particularly given the disproportionately undersurveyed nature of the region's parasite fauna relative to total potential host richness [63].

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List of figures

Fig. 1: Species of *Psettarium* described in this study. A: *Psettarium pandora* n. sp. ex *Ostracion cubicus*, holotype, dorsal view. B: *Psettarium hustoni* n. sp. ex *Arothron nigropunctatus*, holotype, ventral view. C: *Psettarium martini* n. sp. ex *Arothron stellatus*, holotype, dorsal view. D: *Psettarium yoshidai* n. sp. ex *Arothron mappa*, holotype, dorsal view. Vitelline follicles are excluded for all except *P. hustoni* n. sp. Scale-bars: A, B & D, 300 µm; C, 500 µm.

Fig. 2: Terminal genitalia of species of *Psettarium* described in this study, with paths of vitelline duct shown in dotted line. A: *Psettarium pandora* n. sp. ex *Ostracion cubicus*, holotype, dorsal view. B: *Psettarium hustoni* n. sp. ex *Arothron nigropunctatus*, holotype, ventral view. C: *Psettarium martini* n. sp. ex *Arothron stellatus*, holotype, dorsal view. D: *Psettarium yoshidai* n. sp. ex *Arothron mappa*, holotype, dorsal view. Abbreviations: CS- cirrus sac; FGP- female genital pore; MP- male protuberance; Od- oviduct; Oo- oötype; Ov- ovary; PT- posterior testis; Ut- uterus; VD- vas deferens. Scale-bars: A, B & D, 100 µm; C, 200 µm.

Fig. 3: *Psettarium hawaiiense* n. comb. A: Whole worm, dorsal view. Vitelline follicles excluded to prevent obscuring anatomical features. B: Terminal genitalia, dorsal view. C: Fresh egg trapped in liver tissue, showing mild fibrocytic response. Abbreviations: FGP- female genital pore; MP- male protuberance; Od- oviduct; Oo- oötype; Ov- ovary; PT- posterior testis; SV- seminal vesicle; Ut- uterus; VD- vas deferens; VtD- vitelline duct. Scale-bars: A, 250 µm; B, 200 µm; C, 50 µm.

Fig. 4: Eggs of tetraodontid-infecting species of *Psettarium*, photographed *in situ*. A: Eggs of *Psettarium hawaiiense* n. comb. (larger) & *P. nolani* (smaller), ex liver tissue *Arothron hispidus*. B: Eggs of *Psettarium hustoni* n. sp., ex gills *Arothron nigropunctatus*. C: Egg of *Psettarium pulchellum*, ex gills *Arothron manilensis*. D: Eggs of *Psettarium martini* n. sp., ex gills *Arothron stellatus*. E: Eggs of *Psettarium yoshidai* n. sp., ex gills *Arothron mappa*. Scale-bars: A, B, D & E, 50 µm; C, 20 µm.

Fig. 5: Wet mount of gill filament of *Arothron nigropunctatus*, showing a live specimen of *Psettarium hustoni* n. sp. in the afferent artery and eggs trapped in the lamellae. Scale-bar: 400 µm.

Fig. 6: Mounted specimens of *Psettarium nolani* encountered in this study. A/B: Ex *Arothron hispidus* from Gold Coast (A; dorsal view) & Lizard Island (B; ventral view). C/D: Ex *Arothron manilensis* from Moreton Bay (C; dorsal view) & Mackay (D; dorsal view). E: Ex *Arothron stellatus* from Moreton Bay, dorsal view. F: Ex *Arothron meleagris* from Moorea, French Polynesia, dorsal view. Scale-bars: A, B, E & F, 200 µm; C-D, 100 µm.

Fig. 7: Mounted specimens of *Psettarium pulchellum* encountered in this study, all ex *Arothron manilensis* from Lizard Island (A; ventral view), Moreton Bay (B; dorsal view), Mackay (C; dorsal view) & Bali, Indonesia (D; dorsal view). Scale-bar: 200 µm.

Fig. 8: A. Wet-mounts of aporocotylid eggs in various host tissues (all ex *Arothron hispidus*). A. Squashed cyst from mesenteric lining, showing mass of degraded aporocotylid (primarily *Psettarium nolani*) eggs. B. Squashed cyst from wall of mesenteric vessel, showing a mix of dead and live eggs (all *P. nolani*) and worm excreta (dark patches). C. Liver tissue showing masses of live and dead eggs (mix of *P. hawaiiense* n. comb. and *P. nolani*). D. Squash of spleen showing live and degraded eggs of *P. nolani* surrounded by host immune cells. E. Externa of gallbladder showing free and accumulated eggs of *P. hawaiiense* n. comb., the squashed accumulation also showing degraded host immune cells and potentially worm

excreta. F. Gill filament showing eggs of *P. hawaiiense* n. comb. trapped in lamellar tissue. G. Live egg of *P. nolani* in faecal matter obtained from within gut of infected host. H. Live miracidium (species indeterminate) seen in water which held a host fish for 4 hrs. Scale-bars: A, B, D & F, 50 µm; C & E, 150 µm; G-H, 20 µm.

Fig. 9: Histopathological sections of *Arothron hispidus* intestines, stained with Masson's trichrome. A. Mucosal layer of intestine, with numerous individual and accumulated aporocotylid eggs, including possible granulomatous response (far left and right of image). B/C. Individual and small accumulations of aporocotylid eggs close to or contacting the laminar epithelial layer of the intestine, accompanied by host inflammatory immune response. Some evidence of collagen (stained blue) degradation can be observed, along with pervasion of the epithelial layer (bottom left of Image C). D. A single aporocotylid egg, having broken through the laminar epithelial layer. Scale-bars: A, 100 µm; B-D, 50 µm.

Fig. 10: Phylogram based on analyses of the ITS2 rDNA region, showing relationships between species of tetraodontiform-infecting aporocotylids, with locality codes in parentheses. Posterior probabilities are shown above the nodes and bootstrap support values below, with values <80 not shown. Legend for locality codes: BL- Bali; FP- Moorea, French Polynesia; FR- Flora Reef, Cairns; GC- Gold Coast; HI- Heron Island; LI- Lizard Island; MB- Moreton Bay; MK- Mackay.

Fig. 11: Phylogram based on analyses of the 28S rDNA region, showing relationships of all aporocotylid species for which 28S rDNA data are available, with posterior probabilities above the nodes and bootstrap support values below, and values <75 not shown.

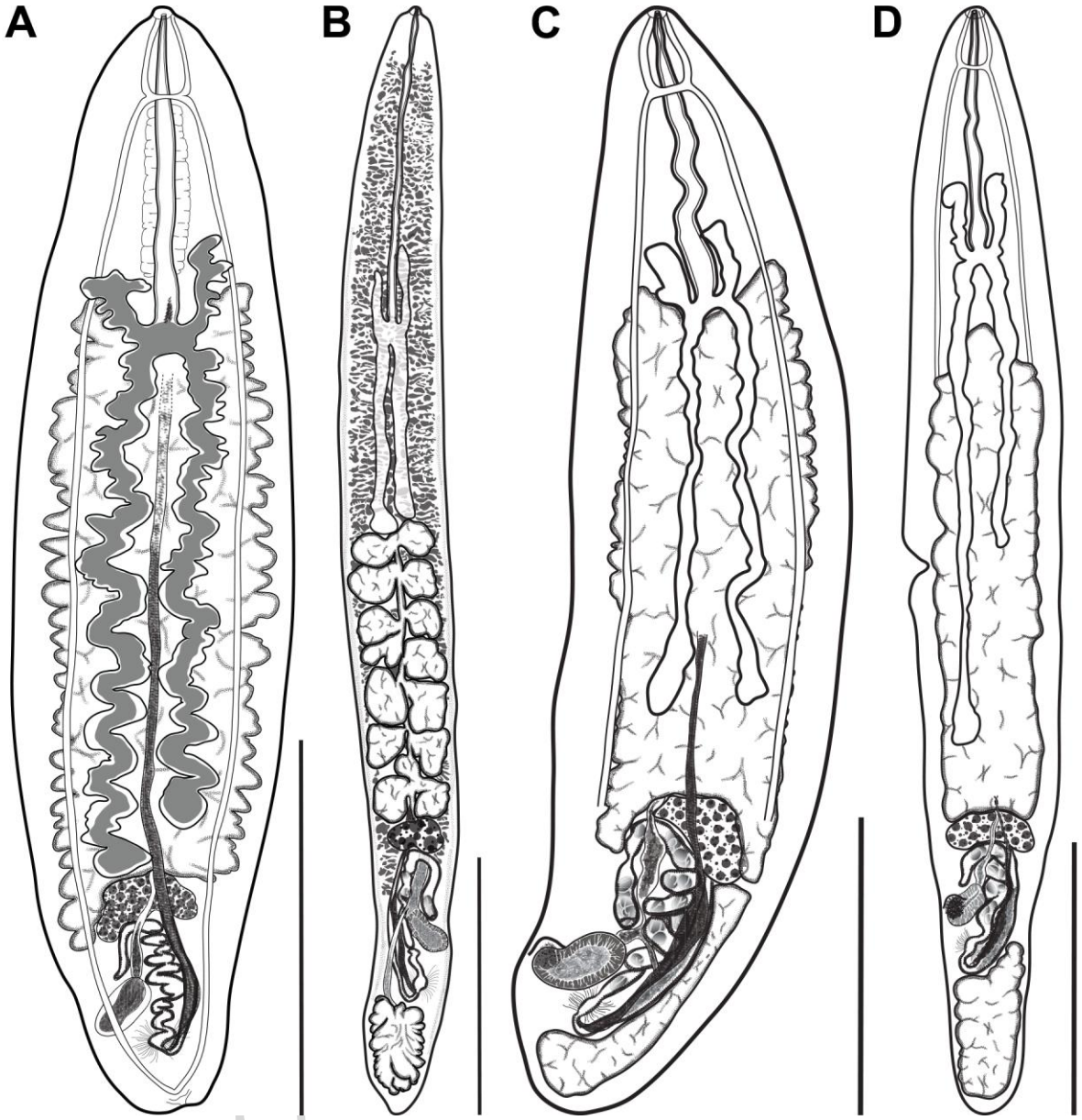
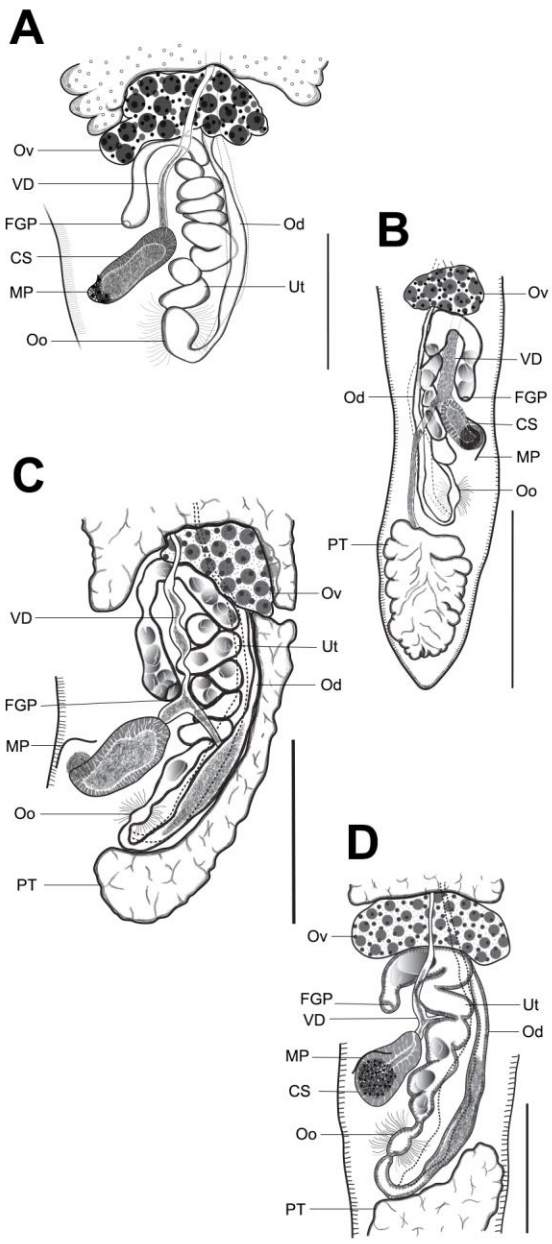


Fig. 1



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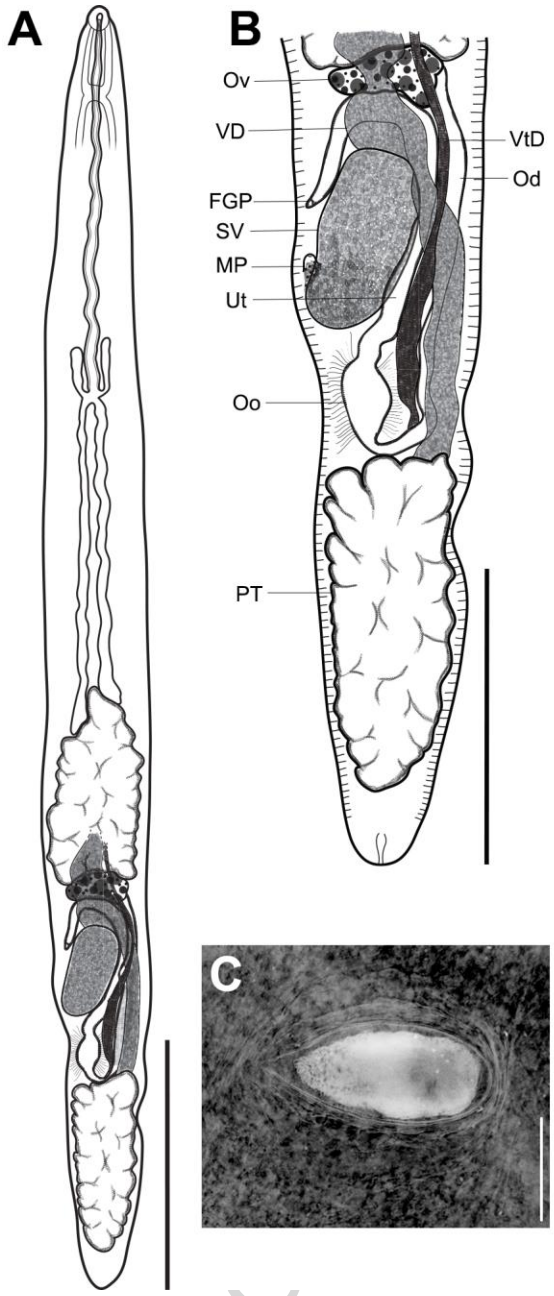


Fig. 3

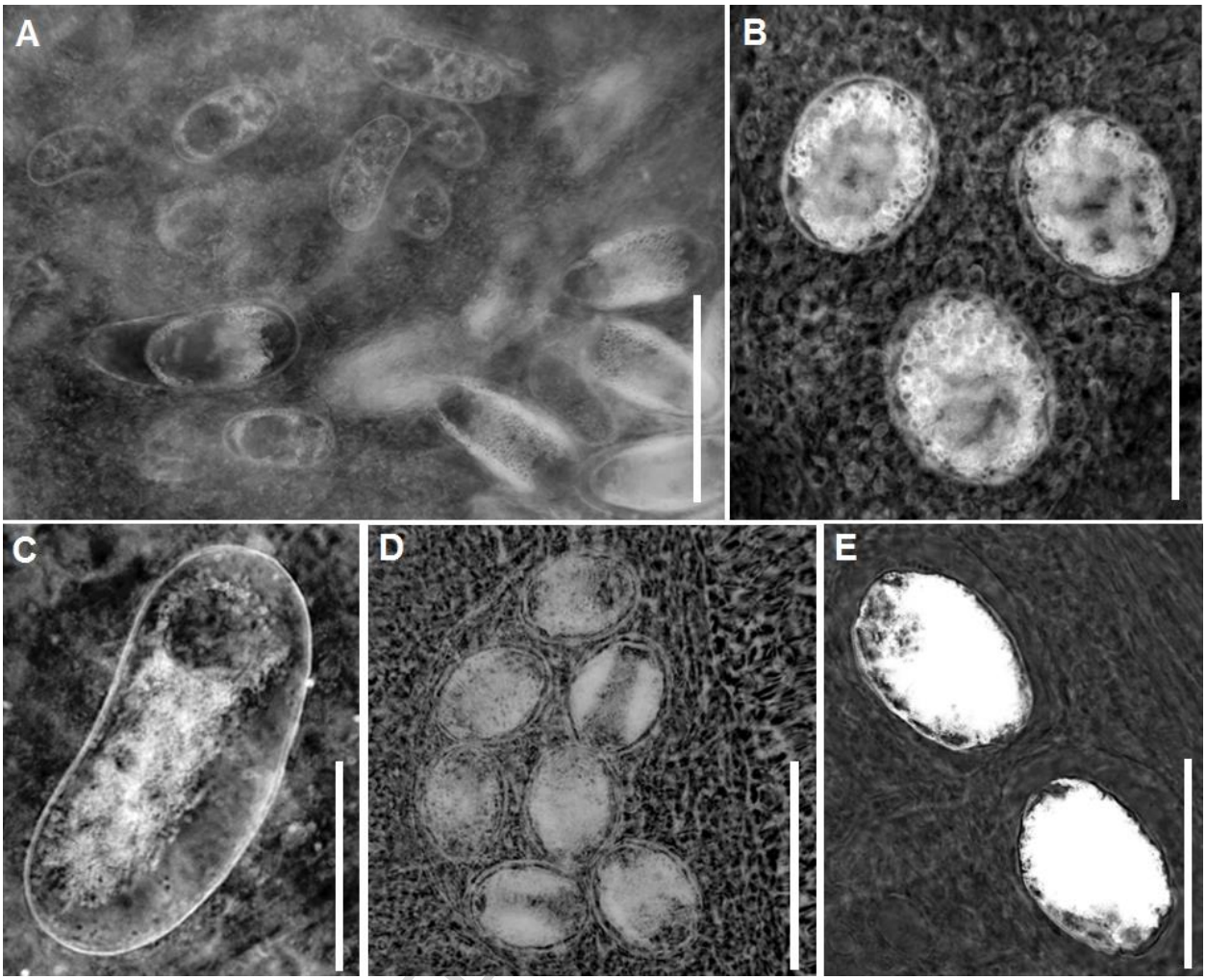
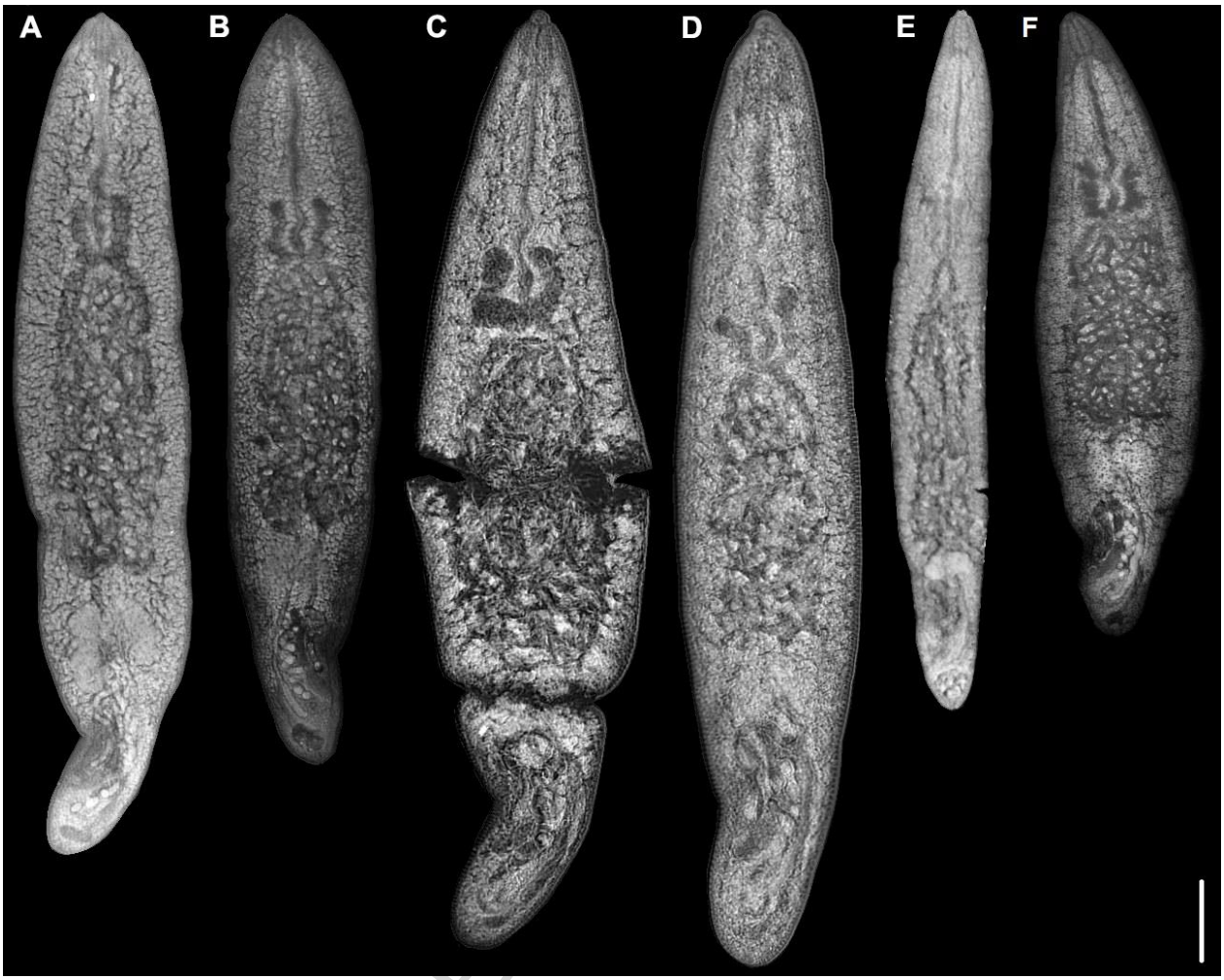


Fig. 4

Fig. 5



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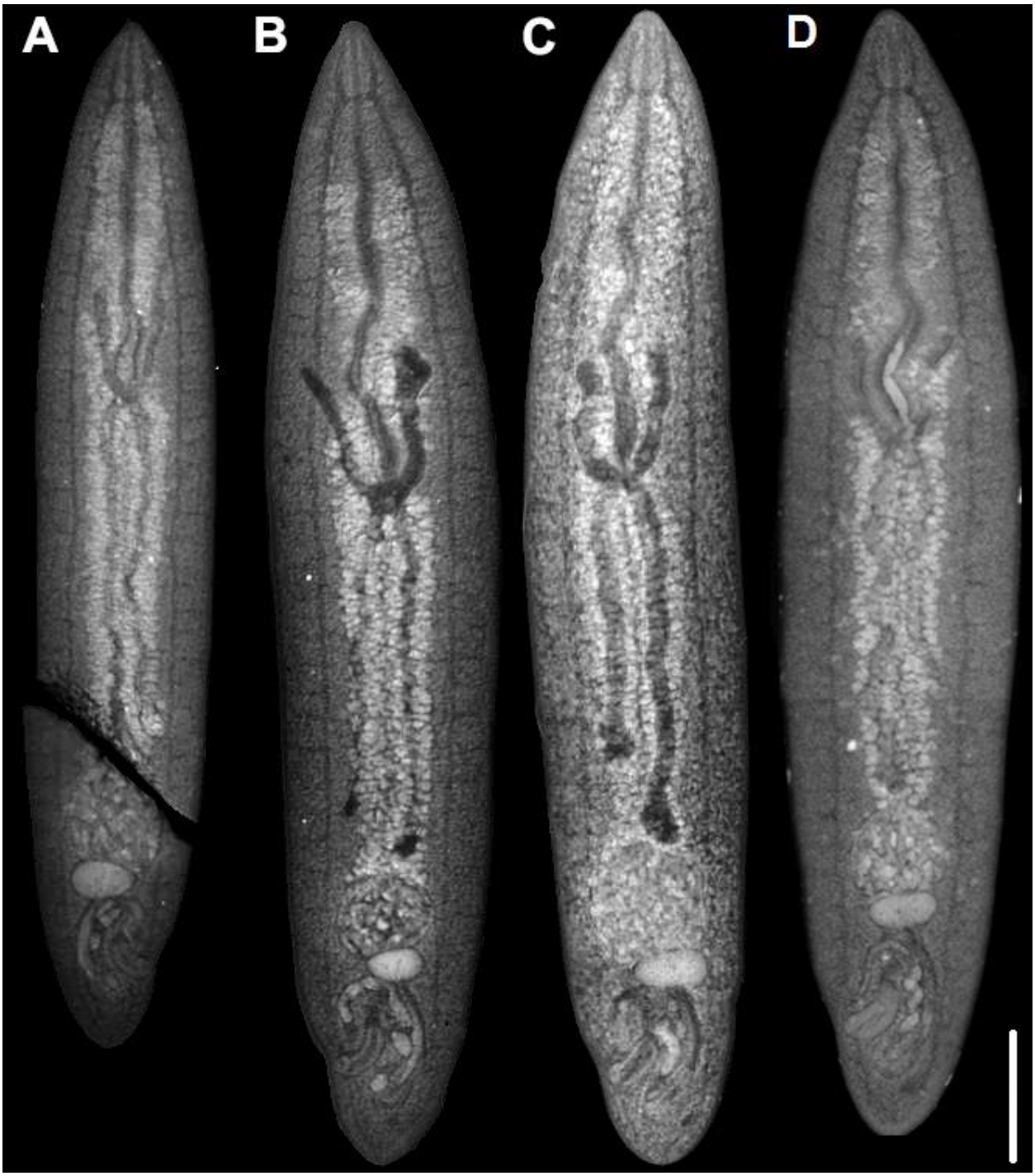

Fig. 7

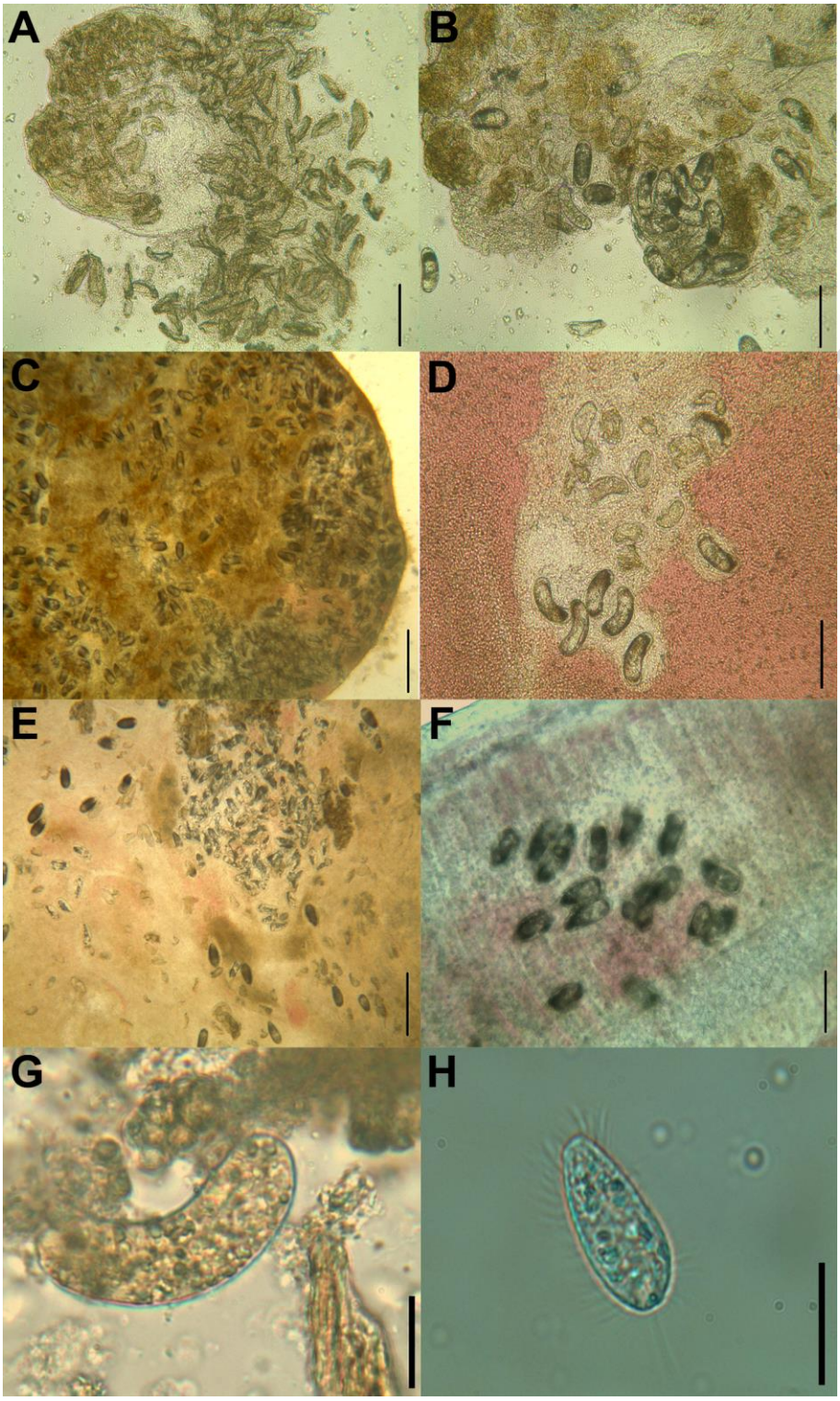

Fig. 8

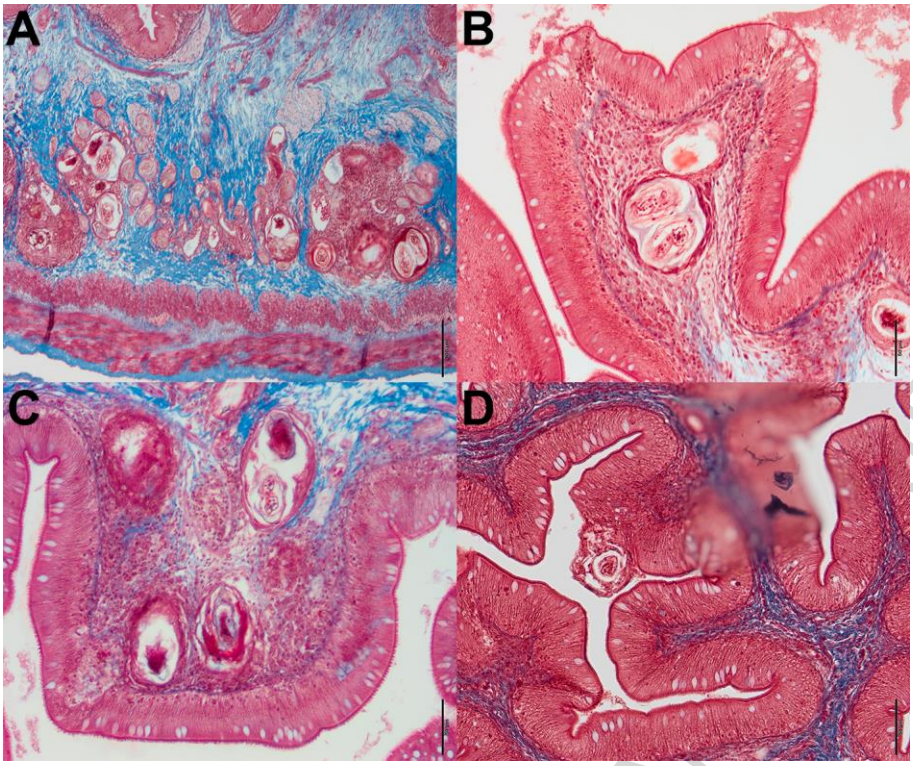

Fig. 9

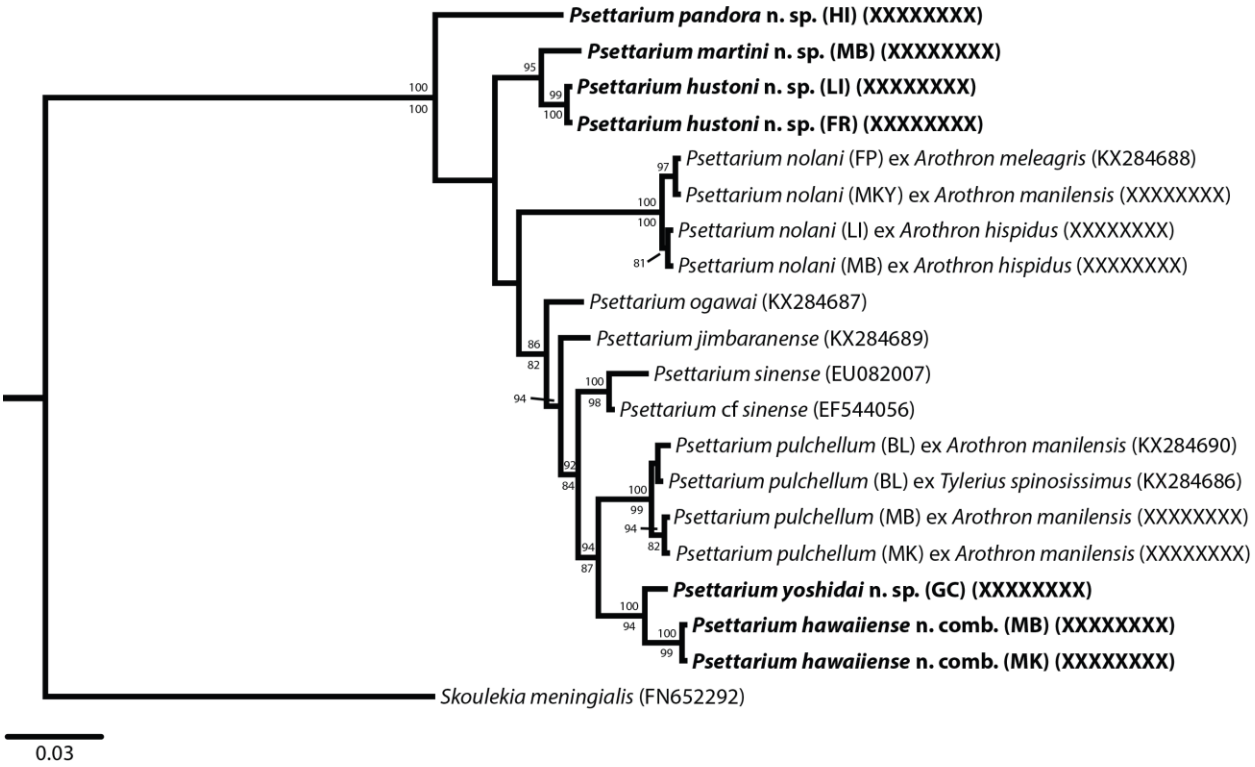

Fig. 10

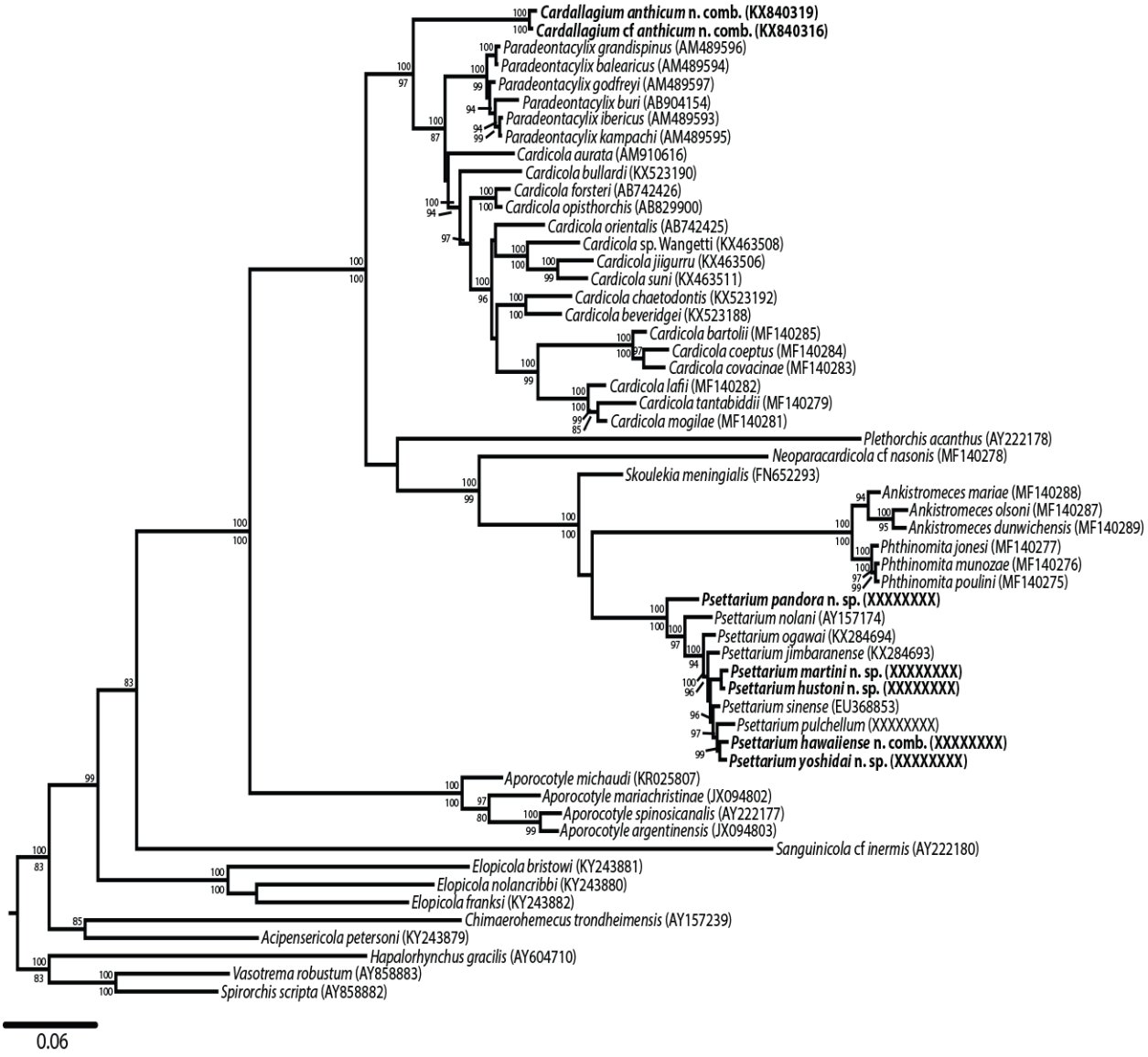

Fig. 11

Table 1: GenBank accession numbers and number of sequence replicates for partial 28S and ITS2 rDNA sequences generated during this study.

Species	GenBank accession no.	
	28S rDNA	ITS2 rDNA
Psettarium hawaiiense n. comb. ex Mackay		XXXXXXXX (2)
Psettarium hawaiiense n. comb. ex Moreton Bay	XXXXXXXX (1)	XXXXXXXX (1)
Psettarium hustoni n. sp. ex Flora Reef	XXXXXXXX (1)	XXXXXXXX (2)
Psettarium hustoni n. sp. ex Lizard Island		XXXXXXXX (1)
Psettarium martini n. sp. ex Gold Coast	XXXXXXXX (1)	XXXXXXXX (1)
Psettarium nolani ex Gold Coast	XXXXXXXX (1)	XXXXXXXX (3)
Psettarium nolani ex Mackay		XXXXXXXX (1)
Psettarium nolani ex Lizard Island		XXXXXXXX (1)
Psettarium pandora n. sp. ex Heron Island	XXXXXXXX (2)	XXXXXXXX (2)
Psettarium pulchellum ex Mackay		XXXXXXXX (1)
Psettarium pulchellum ex Moreton Bay	XXXXXXXX (2)	XXXXXXXX (3)
Psettarium yoshidai n. sp. ex Gold Coast	XXXXXXXX (1)	XXXXXXXX (1)

Table 2 Sequence data from GenBank included in this study. Hosts marked with an asterisk (*) are invertebrate intermediate hosts.

Species	**Host species**	**GenBank accession no.**		**Reference**
		28S rDNA	ITS2 rDNA	
Aporocotylidae Odhner, 1912				
Acipensericola petersoni Bullard, Snyder, Jensen & Overstreet, 2008	*Polyodon spathula* (Walbaum)	KY243879		[74]
Ankistromeces dunwichensis Nolan & Cribb, 2006	*Siganus fuscescens* (Houttuyn)	MF140289	-	[37]
Ankistromeces mariae Nolan & Cribb, 2004	*Meuschenia freycineti* (Quoy & Gaimard)	MF140288	-	[37]
Ankistromeces olsoni Nolan & Cribb, 2006	*Siganus fuscescens*	MF140287	-	[37]
Aporocotyle argentinensis Smith, 1969	*Merluccius hubbsi* Marini	JX094803	-	[66]
Aporocotyle mariachristinae Hernández–Orts, Alama-Bermejo, Carillo, García, Crespo, Raga & Montero, 2012	*Genypterus blacodes* (Forster)	JX094802	-	[66]
Aporocotyle michaudi Santoro, Cipriani, Pankov & Lawton, 2015	*Trematomus bernacchii* Boulenger	KR025807	-	[67]
Aporocotyle spinosicanalis Williams, 1958	*Merluccius merluccius* (Linnaeus)	AY222177	-	[71]
Cardallagium anthicum (Bullard & Overstreet, 2006)	*Rachycentron canadum* Linnaeus	KX840316	-	[31]
Cardallagium cf *anthicum*	*Rachycentron canadum*	KX840319	-	[31]
Cardicola aurata Holzer, Montero, Repulles, Nolan, Sitja–Bobadilla, Alvarez–Pellitero, Zarza & Raga, 2008	*Sparus aurata* Geoffroy Saint-Hilaire	AM910616	-	[65]
Cardicola bartolii Nolan & Cribb, 2006	*Siganus lineatus* (Valenciennes)	MF140285	-	[37]
Cardicola beveridgei Nolan, Miller, Cutmore, Cantacessi & Cribb, 2014	*Lutjanus bohar* Forsskål	KX523188	-	[41]
Cardicola bullardi Nolan, Miller, Cutmore, Cantacessi & Cribb, 2014	*Scomberomorus munroi* Collette & Russo	KX523190	-	[41]
Cardicola chaetodontis Yamaguti, 1970	*Chaetodon plebeius* Cuvier	KX523192	-	[64]
Cardicola coeptus Nolan & Cribb, 2006	*Siganus punctatus* Schneider & Forster	MF140284	-	[37]
Cardicola covacinae Nolan & Cribb, 2006	*Siganus punctatus*	MF140283	-	[37]
Cardicola forsteri Cribb, Daintith & Munday, 2000	*Thunnus maccoyii* (Castelnau)	AB742426	-	[73]
Cardicola jiigurru Yong, Cutmore, Miller, Wee & Cribb, 2016	*Chanos chanos* Forsskål	KX463506	-	[41]

Cardicola lafii Nolan & Cribb, 2006	*Siganus fuscescens*	MF140282	-	[37]
Cardicola mogilae Brooks, Cutmore, Yong & Cribb, 2017	*Siganus fuscescens*	MF140281	-	[37]
Cardicola opisthorchis Ogawa, Ishimaru, Shirakashi, Takami & Grabner, 2011	*Terebella* sp.*	AB829900	-	[69]
Cardicola orientalis Ogawa, Tanaka, Sugihara & Takami, 2009	*Thunnus maccoyii*	AB742425	-	[73]
Cardicola suni Yong, Cutmore, Miller, Wee & Cribb, 2016	*Chanos chanos*	KX463511	-	[41]
Cardicola tantabiddii Nolan & Cribb, 2006	*Siganus fuscescens*	MF140279	-	[37]
Cardicola sp. Wangetti	*Chanos chanos*	KX463508	-	[41]
Chimaerohemecus trondheimensis Van der Land, 1967	*Chimaera monstrosa* Linnaeus	AY157239	-	
Elopicola bristowi Orélis-Ribeiro, Halanych, Dang, Bakenhaster, Arias & Bullard, 2017	*Elops hawaiensis* Regan	KY243881	-	[73]
Elopicola franksi Orélis-Ribeiro, Halanych, Dang, Bakenhaster, Arias & Bullard, 2017	*Megalops atlanticus* Valenciennes	KY243882	-	[73]
Elopicola nolancribbi Bullard, 2014	*Elops saurus* Linnaeus	KY243880	-	[73]
Neoparacardicola cf. *nasonis* Yamaguti, 1970	*Naso unicornis* (Forsskål)	MF140278	-	[37]
Paradeontacylix balearicus Repullés-Albelda, Montero, Holzer, Ogawa, Hutson & Raga, 2008	*Seriola dumerili* (Risso)	AM489594	-	[51]
Paradeontacylix buri Ogawa, Akiyama & Grabner, 2015	*Seriola quinqueradiata* Temminck & Schlegel	AB904154	-	[68]
Paradeontacylix godfreyi Hutson & Whittington, 2006	*Seriola lalandi* (Valenciennes)	AM489597	-	[51]
Paradeontacylix grandispinus Ogawa & Egusa, 1986	*Seriola dumerili*	AM489596	-	[51]
Paradeontacylix ibericus Repulles-Albelda, Montero, Holzer, Ogawa, Hutson & Raga, 2008	*Seriola dumerili*	AM489593	-	[51]
Paradeontacylix kampachi Ogawa & Egusa, 1986	*Seriola dumerili*	AM489595	-	[51]
Plethorchis acanthus Martin, 1975	*Mugil cephalus* Linnaeus	AY222178	-	[71]
Psettarium jimbaranense Yong, Cutmore, Bray, Miller, Semarariana, Palm & Cribb, 2016	*Arothron reticularis* Bloch & Schneider	KX284693	KX284689	[8]

Psettarium nolani (Bray, Cribb & Littlewood, 2012)	*Arothron meleagris* (Anonymous)	AY157174	KX284688	[8, 71]
Psettarium ogawai Yong, Cutmore, Bray, Miller, Semarariana, Palm & Cribb, 2016	*Arothron reticularis*	KX284694	KX284687	[8]
Psettarium pulchellum Yong, Cutmore, Bray, Miller, Semarariana, Palm & Cribb, 2016	*Arothron manilensis* (de Procé)	KX284692	KX284690	[8]
Psettarium pulchellum	*Tylerius spinosissimus* Regan	-	KX284686	[8]
Psettarium sinense (as *Paradeontacylix sinensis*) (Liu, 1997)	*Takifugu rubripes* (Temminck & Schlegel)	EU368853	EU082007	Na
Psettarium cf *sinense*	*Takifugu niphobles* (Jordan & Snyder)	-	EF544056	Na
Sanguinicola cf *inermis* Plehn, 1905	*Lymnaea stagnalis**	AY222180	-	[71]
Skoulekia meningialis Alama-Bermejo, Montero, Raga & Holzer, 2011	*Diplodus vulgaris* (Geoffroy Saint-Hilaire)	FN652293	FN652292	[35]
Spirorchiidae Stunkard, 1921				
Hapalorhynchus gracilis Stunkard, 1922	*Chelydra serpentina*	AY604710	-	[72]
Spirorchis scripta Stunkard, 1923	*Chrysemys picta*	AY858882	-	[70]
Vasotrema robustum Stunkard, 1928	*Apalone spinifera*	AY858883	-	[70]

Abbreviation: na, not available

Table 3 Total pairwise uncorrected p-differences among sequences of tetraodontiform-infecting species of *Psettarium*; partial 28S sequences above and ITS2 sequences below the diagonal. Abbreviations: BL- Bali; FP- French Polynesia; FR- Flora Reef; GC- Gold Coast; HI- Heron Island; LI- Lizard Island; MB- Moreton Bay; MK- Mackay.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. *Psettarium jimbaranense* KX284689	-	14	-	22	-	-	-	11	14	16	-	17	15	-	28	-	-	-	41
2. *Psettarium ogawai* KX284687	5	-	-	20	-	-	-	11	14	14	-	17	15	-	30	-	-	-	40
3. *Psettarium* sp cf *sinense* EF544056	6	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4. *Psettarium pulchellum* BL KX284686	7	8	7	-	-	-	-	17	16	18	-	19	21	-	30	-	-	-	49
5. *Psettarium pulchellum* BL KX284690	8	9	8	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6. *Psettarium pulchellum* MB	8	9	8	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7. *Psettarium pulchellum* MK	8	9	8	1	2	0	-	-	-	-	-	-	-	-	-	-	-	-	-
8. *Psettarium sinense* EU082007	10	11	4	11	12	12	12	-	9	11	-	12	10	-	27	-	-	-	42
9. *Psettarium yoshidai* n. sp. GC	10	11	10	9	10	10	10	14	-	8	-	14	12	-	24	-	-	-	41
10. *Psettarium hawaiiense* n. comb. MB	12	13	12	9	10	10	10	16	4	-	-	15	13	-	28	-	-	-	39
11. *Psettarium hawaiiense* n. comb. MK	12	13	12	9	10	10	10	16	4	0	-	-	-	-	-	-	-	-	-
12. *Psettarium martini* n. sp. GC	12	12	14	11	12	12	12	18	16	16	16	-	6	-	31	-	-	-	46
13. *Psettarium hustoni* n. sp. LI	13	12	15	14	15	15	15	19	17	17	17	6	-	-	31	-	-	-	45
14. *Psettarium hustoni* n. sp. FR	13	12	15	14	15	15	15	19	17	17	17	6	0	-	-	-	-	-	-
15. *Psettarium nolani* GC	15	14	17	16	17	17	17	21	17	19	19	18	16	16	-	-	-	-	39
16. *Psettarium nolani* LI	15	14	17	16	17	17	17	21	17	19	19	18	16	16	0	-	-	-	-
17. *Psettarium nolani* FP KX284688	16	15	18	17	18	18	18	22	18	20	20	19	17	17	1	1	-	-	-
18. *Psettarium nolani* MK	16	15	18	17	18	18	18	22	18	20	20	19	17	17	1	1	0	-	-
19. *Psettarium pandora* n. sp. HI	20	19	22	21	22	22	22	26	22	26	26	20	20	20	23	23	24	24	-