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Description of three species of *Isorchis* (Digenea: Atractotrematidae) from Australia

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Abstract

Three species of *Isorchis* Durio and Manter, 1969 are described from Australian waters. *Isorchis megas* sp. nov. is described from the spotbanded scat, *Selenotoca multifasciata* (Richardson), off Western Australia (WA) and Northern Territory (NT); *Isorchis currani* sp. nov. is described from *S. multifasciata* off NT; and *Isorchis anomalus* sp. nov. is described from the milkfish, *Chanos chanos* Forsskål, off WA. *Isorchis megas* sp. nov. can be differentiated from the other species of *Isorchis* by possessing a single, large egg that is greater than 20% of the body length; having a shorter body (the largest specimen is less than 500 μm); and utilizing a scatophagid rather than a chanid host. *Isorchis currani* sp. nov. can be differentiated from species of *Isorchis* other than *I. megas* sp. nov. by utilizing a scatophagid rather than a chanid host; it is differentiated from *I. megas* sp. nov. in having eggs that are 11−15% of the body length. *Isorchis anomalus* sp. nov. can be differentiated from all other species of *Isorchis* in possessing an irregular shaped genital pore rather than one that is circular to oblong. A Bayesian inference analysis of partial 28S rDNA sequences of the three new species of *Isorchis* and 30 other haploporoids revealed 1) the monophyly of the Atractotrematidae Yamaguti, 1939, 2) the two species of *Isorchis* infecting *S. multifasciata* were each other's closest relative, and 3) that *Isorchis* was most closely related to *Pseudomegasolena* Machida and Komiya, 1976 rather than *Atractotrema* Goto and Ozaki, 1929 although sequence data are not yet available for a member of *Pseudisorchis* Ahmad, 1985.

Keywords

Chanos, trematode, Haploporidae, Haploporoidea, Scatophagidae, phylogeny

Introduction

Jones (2005) considered the Haploporoidea Nicoll, 1914 to comprise the Atractotrematidae Yamaguti, 1939 and the Haploporidae Nicoll, 1914. Members of the superfamily utilize the alimentary tract or gall bladder of marine (Atractotrematidae and Haploporidae), estuarine (Atractotrematidae and Haploporidae), and freshwater (Haploporidae) herbivorous and omnivorous fishes (e.g., Overstreet and Curran 2005a,b; Bray *et al.* 2014). Members of the superfamily are morphologically united by the presence of a hermaphroditic sac enclosing the terminal portion of the male and female reproductive structures. Olson *et al.* (2003) transferred both families into the superfamily Gorgoderoidea Looss, 1901 based on molecular analysis of 18S and 28S rDNA sequences, but remarked that the two families were among the most labile. Curran *et al.* (2006) utilized the analysis of 28S rDNA sequences to reinstate the Haploporoidea.

Durio and Manter (1969) and Ahmad (1985) considered the Atractotrematidae a junior synonym of the Haploporidae, but that view has not prevailed on a morphological basis (Yamaguti 1971; Overstreet and Curran 2005a,b) or by molecular analysis (e.g., Blasco-Costa *et al.* 2009; Pulis and Overstreet 2013; Andres *et al.* 2014). However, Overstreet and Curran (2005b) considered the status of the Atractotrematidae as tentative because the family is depauperate (containing eight species in four genera) and has yet to have a life-cycle published. Furthermore, molecular data had not been provided for any atractotrematid taxon since the study by Olson *et al.* (2003) whereas several studies (e.g., Blasco-Costa *et al.* 2009; Pulis and Overstreet 2013; Bray *et al.* 2014; Andres *et al.* 2015) have improved our understanding of the interrelationships of the Haploporidae.

The Atractotrematidae contains *Atractotrema* Goto and Ozaki, 1929, *Isorchis* Durio and Manter, 1969, *Pseudisorchis* Ahmad, 1985, and *Pseudomegasolena* Machida and Kamiya,

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1976. *Isorchis* is differentiated from *Atractotrema* in having testes located in the hindbody; from *Pseudisorchis* in having the ovary at the level of the testes; and from *Pseudomegasolena* in having a fusiform rather than circular body shape as well as parasitizing chanid rather than scarid hosts (Overstreet and Curran 2005b). *Isorchis* was established by Durio and Manter (1969) for *I. parvus* from *Chanos chanos* (Forsskål) off New Caledonia. Zhukov (1972) erected *Krusadaitrema* Zhukov, 1972 for *Krusadaitrema chanosi* Zhukov, 1972 collected from *C. chanos* off Krusadai Island, India. Ahmad (1985) described *Isorchis skrjabini* Ahmad, 1985 from *C. chanos* from the Arabian Sea off Goa, India. He also considered *Krusadaitrema* a junior synonym of *Isorchis* and transferred *Krusadaitrema chanosi* to *Isorchis* as *Isorchis chanosi* (Zhukov, 1972) Ahmad, 1985. Therefore, prior to this study, *Isorchis* contained three species, all of which were described from *C. chanos*. We describe three additional species of *Isorchis* from Australia, provide sequence comparisons of the ribosomal DNA (rDNA) internal transcribed spacer region (= ITS1, 5.8S, and ITS2) and the 28S of the three new species, and conduct a Bayesian inference (BI) analysis of the new species with 30 other haploporoids to test the monophyly of the Atractotrematidae.

Materials and Methods

During February 2010, specimens of *Isorchis* were collected from the milkfish, *Chanos chanos*, off Learmouth, Western Australia (WA), and from *Selenotoca multifasciata* (Rishardson) off Dampier, WA, and Fannie Bay, Northern Territory, Australia. Hosts were collected with a cast-net. Specific fish names follow those given by FishBase (Froese and Pauly 2015). Haploporoids were isolated following the method similar to that of Cribb and Bray (2010) for gastrointestinal species but skipping the initial examination under a dissecting microscope because of the large volume of intestinal contents. The worms were rinsed and cleaned in a container with saline and examined briefly. Some specimens were placed directly into cool 95% molecular grade ethanol, but most of the worms were killed by pouring hot (not boiling) water over them and then preserved in 70% molecular grade ethanol. Worms were stained in Mayer's haematoxylin, dehydrated in a graded ethanol series, cleared in methyl salicylate, and mounted permanently in Dammar gum. Measurements were made using a compound microscope equipped with differential interference contrast, a Canon EOS Rebel T1i camera, and calibrated digital software (iSolutions Lite ©). All measurements are in micrometres; data for the holotypes are presented in the corresponding descriptions. Terminology of the hermaphroditic sac and its structures follows the terms used by Pulis and Overstreet (2013). Museum abbreviations are as follows: NTM, Museum and Art Gallery of the Northern Territory, Darwin, Australia; USNM, Smithsonian National Museum of Natural History, Washington, DC, USA; and WAM, Western Australian Museum, Perth, Western Australia, Australia.

Genomic DNA was extracted from three entire specimens for each of the new species that were either fixed in cool 95% ethanol or heat killed worms in 70% ethanol using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the instructions provided. DNA fragments ca 2,400 base pairs (bp) long, comprising the 3' end of the 18S nuclear rRNA gene, the entire ITS region, and the 5' end of the 28S rRNA gene (including variable domains D1–D3), were amplified from the extracted DNA by polymerase chain reaction (PCR) on a PTC-200 Peltier Thermal Cycler using forward primer ITSF (5'-CGCCCGTCGCTACTACCGATTG-3') and reverse primer 1500R (5'-GCTATCCTGAGGGAAACTTCG-3'). These PCR primers and multiple internal primers were used in sequencing reactions. The internal forward primers were DIGL2 (5'-AAGCATATCACTAAGCGG-3'), 300F (5'-CAAGTACCGTGAGGGAAAGTTG-3'), and 900F (5'-CCGTCTTGAAACACGGACCAAG-3') and the internal reverse primers were 300R (5'-CAACTTTCCCTCACG-GTACTTG-3'), DIGL2R (5'-CCGCTTAGTGATATGCTT-3'), and ECD2 (5'-CTTGGTCCGTGTTTCAAGACGGG-3'). The resulting PCR products were excised from PCR gel using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, California, USA) following the kit instructions, cycle-sequenced using ABI BigDye™ chemistry (Applied Biosystems, Inc., Carlsbad, California, USA), ethanol-precipitated, and run on an ABI 3130 Genetic Analyzer™. Contiguous sequences from the species were assembled using Sequencher™ (GeneCodes Corp., Ann Arbor, Michigan, USA, Version 5.0) and submitted to GenBank. Previously published 28S ribosomal RNA gene sequences of species of and close to the Haploporoidea were used for comparison (see Table I for accession numbers and host information) with newly submitted sequences.The sequences were aligned using MAFFT version 6.611b (Katoh *et al.* 2005) with 1,000 cycles of iterative refinement and the *genafpair* algorithm. The alignment was masked with ZORRO (Wu *et al.* 2012) using default settings, positions with confidence scores <0.4 were excluded and the alignment was trimmed to the shortest sequence on both 5' and 3' ends in BioEdit, ver. 7.1.3.0. (Hall 1999). The resulting 28S alignment utilized 2 species of *Paragonimus* Braun, 1899 and 30 haploporoids with *Paragonimus westermani* Kerbert, 1878 as the outgroup based on its phylogenetic position relative to the Haploporoidea (Olson *et al.* 2003) and to be consistent with previous analyses (Pulis *et al.* 2013, Bray *et al.* 2014, Andres *et al.* 2014). Phylogenetic analyses of the data were performed using BI with MrBayes 3.1.2 software (Huelsenbeck and Ronquist 2001). The best nucleotide substitution model was estimated with jModeltest-2 (Darriba *et al.* 2012) as general time reversible with estimates of invariant sites and gamma-distributed among site-rate variation (GTR + I + Γ). The following model parameters were used in MrBayes for the analysis: nst = 6, rates = invgamma, ngen = 1,000,000, and samplefreq = 100. Burn-in value was 2,500 estimated by plotting the log-probabilities against generation and visualizing plateau in parameter values (sump burnin = 2,500), and nodal support was estimated by posterior probabilities

Table I. Sequences from GenBank used for phylogenetic analysis in this study

Family	Species	Host	GenBank	Reference
Atractotrematidae	*Atractotrema sigani* Durio and Manter, 1969	*Siganus lineatus* (Valenciennes)	AY222267	Olson *et al.* (2003)
	Pseudomegasolena ishigakiense Machida and Kamiya, 1976	*Scarus rivulatus* Valenciennes	AY222266	Olson *et al.* (2003)
'Cadenatellinae'	*Cadenatella isuzumi* Machida, 1993	*Kyphosus vaigiensis* Quoy and Gaimard	FJ788497	Bray *et al.* (2009)
	Cadenatella pacifica (Yamaguti, 1970)	*Kyphosus vaigiensis*	FJ788498	Bray *et al.* (2009)
Haploporidae	*Hapladena nasonis* Yamaguti, 1970	*Naso unicornis* (Forsskål)	AY222265	Olson *et al.* (2003)
	Dicrogaster contracta Looss, 1902	*Liza aurata* (Risso)	FJ211261	Blasco-Costa *et al.* (2009a)
	Dicrogaster perpusilla Looss, 1902	*Liza ramada* (Risso)	FJ211238	Blasco-Costa *et al.* (2009a)
	Forticulcita apiensis Andres, Curran, Fayton, Pulis, and Overstreet, 2015	*Mugil cephalus* Linnaeus	KP761087	Andres *et al.* (2015)
	Forticulcita gibsoni Blasco-Costa, Montero, Balbuena, Raga, and Kostadinova, 2009	*Mugil cephalus*	FJ211239	Blasco-Costa *et al.* (2009a)
	Forticulcita platana Andres, Curran, Fayton, Pulis, and Overstreet, 2015	*Mugil liza* Valenciennes	KP761086	Andres *et al.* (2015)
	Haploporus benedeni (Stossich, 1887)	*Liza ramada*	FJ211237	Blasco-Costa *et al.* (2009a)
	Litosaccus brisbanensis (Martin, 1974)	*Mugil cephalus*	KM253765	Andres *et al.* (2014)
	Lecithobotrys putrescens Looss, 1902	*Liza saliens* (Risso)	FJ211236	Blasco-Costa *et al.* (2009a)
	Ragaia lizae Blasco-Costa, Montero, Gibson, Balbuena, and Kostadinova, 2009	*Liza aurata*	FJ211235	Blasco-Costa *et al.* (2009a)
	Saccocoelium brayi Blasco-Costa, Balbuena, Raga, Kostadinova, and Olson, 2010	*Liza saliens*	FJ211234	Blasco-Costa *et al.* (2009a)
	Saccocoelium cephali Blasco-Costa, Montero, Gibson, Balbuena, Raga and Kostadinova, 2009	*Mugil cephalus*	FJ211233	Blasco-Costa *et al.* (2009a)
	Saccocoelium obesum Looss, 1902	*Liza ramada*	FJ211259	Blasco-Costa *et al.* (2009a)
	Saccocoelium tensum Looss, 1902	*Liza aurata*	FJ211258	Blasco-Costa *et al.* (2009a)
	Saccocoelioides sp.	Poeciliidae Garman	EF032696	Curran *et al.* (2006)
	Capitimitta costata Pulis and Overstreet, 2013	*Selenotoca multifasciata* (Richardson)	KC206497	Pulis and Overstreet (2013)
	Capitimitta darwinensis Pulis and Overstreet, 2013	*Selenotoca multifasciata*	KC206498	Pulis and Overstreet (2013)
	Capitimitta sp.	*Selenotoca multifasciata*	KC206499	Pulis and Overstreet (2013)
	Spiritestis herveyensis Pulis and Overstreet, 2013	*Moolgarda seheli* (Forsskål)	KC206500	Pulis and Overstreet (2013)
	Intromugil alachuaensis Pulis, Fayton, Curran and Overstreet, 2013	*Mugil cephalus*	KC430095	Pulis *et al.* (2013)
	Intromugil mugilicolus (Shireman, 1964)	*Mugil cephalus*	KC430096	Pulis *et al.* (2013)
	Parasaccocoelium haematocheilum Besprozvannykh, Atopkin, Ermolenko and Nikitenko, 2014	*Liza haematocheila* (Temminck and Schlegel)	HF548461	Besprozvannykh *et al.* (2014)
	Parasaccocoelium mugili Zhukov, 1971	*Liza haematocheila*	HF548468	Besprozvannykh *et al.* (2014)
	Parasaccocoelium polyovum Besprozvannykh, Atopkin, Ermolenko, and Nikitenko, 2014	*Liza haematocheila*	HF548474	Besprozvannykh *et al.* (2014)
	Xiha fastigata (Thatcher and Sparks, 1958)	*Mugil cephalus* Linnaeus	KP761088	Andres *et al.* (2015)
Paragonimidae	*Paragonimus westermani* (Kerber, 1878)	*Canis lupus familiaris* Linnaeus	AY116874	Olson *et al.* (2003)
	Paragonimus iloktsuenensis Chen, 1940	*Rattus norvegicus* (Berkenhout, 1769)	AY116875	Olson *et al.* (2003)

(sumt) (Huelsenbeck *et al.* 2001) with all other settings left as default.

All pairwise comparisons of sequence data were made calculated in MEGA ver. 6 (Tamura *et al.* 2013) as p-distances and excluded gaps.

Results

Atractotrematidae Yamaguti, 1939

Isorchis megas sp. nov. (Figs 1–2)

Description based on 7 gravid (4 from off Dampier and 3 from off Darwin) and 6 nongravid (all from off Dampier), wholemounted, unflattened specimens. Body fusiform, tapering posteriorly, 348 long, 147 wide at midbody representing 42% of body length (BL). Forebody 163 long representing 47% of BL. Hindbody 128 long representing 37% of BL. Eyespot pigment primarily dispersed in forebody (dispersed over anterior 3/5 of BL in 1 specimen), more prominent dorsally. Tegumental spines ≤1 long, fine, occurring over entire body surface. Oral sucker terminal, transversely subglobular, 67 long, 85 wide. Ventral sucker subglobular, 57 long, 59 wide. Ratio of oral sucker width to ventral sucker width 1: 0.69. Prepharynx 11 long. Pharynx transversely subglobular, 43 long, 56 wide. Ratio of oral sucker to pharynx width 1:0.66. Oesophagus straight to sinuous, 55 long. Intestinal bifurcation contiguous with level of anterior margin of ventral sucker. Caeca approximately 3.9 times as long as wide, terminating blindly 79 from posterior end; postcaecal space representing 23% of BL.

Testes 2, symmetrical or nearly so, postequatorial or nearly so, at approximately level of ventral sucker, elongate; sinistral testis 69 long, 38 wide; dextral testis 70 long, 37 wide. Postesticular space 32% of BL. External seminal vesicle claviform to sac-like, 38 long, 19 wide, dorsal to ventral sucker. Hermaphroditic sac 45 long, 36 wide representing 13% of BL; containing terminal genitalia; internal seminal vesicle 20 long, 18 wide; prostatic bulb elongate to subglobular; male duct short, uniting with female duct at approximately midlevel to anterior 1/3 of sac; hermaphroditic duct approximately 1/3 length of hermaphroditic sac, curved; diverticula 2, uniting with hermaphroditic duct anteriorly. Genital pore medial, 13 anterior to anterior margin of ventral sucker.

Ovary subglobular to globular, 32 long, 21 wide, intercaecal, ventral to level of caeca, intertesticular, dorsal to ventral sucker. Laurer's canal not observed. Vitellarium follicular; follicles relatively few, 10–18 long, 9–17 wide, extending anteriorly to 110 from anterior margin, extending posteriorly to 64 from posterior margin, interrupted at level of testes in some, contiguous dorsally when not interrupted; vitelline reservoir subglobular, 39 long, 38 wide, slightly overlapping to contiguous with posterior margin of ovary. Uterus restricted to region between vitelline reservoir and hermaphroditic sac. Egg 1, large, 84 long representing 24% of BL, 39 wide.

Excretory vesicle Y-shaped, bifurcating 69 from posterior margin of body, with arms extending to approximately level of pharynx, representing 68% of BL; excretory pore terminal.

Taxonomic summary

Type- and only known host: *Selenotoca multifasciata* (Richardson), spotbanded scat, (Scatophagidae).
Site of infection: Intestine.

Type-locality: off Dampier boat ramp, Western Australia, Australia (20°39′22.5″S, 116°42′25″E); other locality: Doyles boat ramp, Fannie Bay, Darwin, Northern Territory, Australia (12°26′8.7″S, 130°49′56″E).

Specimens deposited: Holotype WAM V8549; paratypes WAM V8550–8552 (n = 3), NTM D000769, D000772, D001328, D001567 (n = 4), USNM 1254765–1254768 (n = 4).

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. KU873015 from 2 entire specimens from Dampier and 1 entire specimen from Darwin.

Etymology: The Greek masculine "*megas*" refers to the large egg size relative to body length.

Remarks

Isorchis megas sp. nov. can be differentiated from the other three species of *Isorchis* based on a shorter body length (the largest individuals <410 μm; see Table II), a shorter testes (less than 100 μm), fewer eggs in mature individuals (always one or less in all of our specimens), an egg length that represents greater than 20% of the body length, and a reduced distribution of vitelline follicles. *Isorchis megas* is further separated from *I. parvus* and *I. skrjabin* in having an intestinal bifurcation closer to the anterior margin of the ventral sucker than to the posterior margin of the pharynx. This species is the first species of *Isorchis* to be described from a non-chanid host.

Isorchis currani sp. nov. (Figs 3–4)

Description based on 6 mature, wholemounted, unflattened specimens. Body fusiform, 591 long, 251 wide at midbody representing 42% of BL. Forebody 260 long representing 44% of BL. Hindbody 248 long representing 42% of BL. Eyespot pigment lightly dispersed in anterior midforebody (more prominent dorsally in 1 specimen). Tegumental spines ≤1 long, fine, dense, occurring over entire body surface. Oral sucker terminal, subglobular, 89 long, 111 wide. Ventral sucker subglobular, 81 long, 75 wide. Ratio of oral sucker to ventral sucker widths 1: 0.68. Prepharynx 27 long. Pharynx globular to subglobular, 68 long, 67 wide. Ratio of oral sucker to pharynx width 1:0.60. Oesophagus straight to sinuous, 76 long. Intestinal bifurcation at or slightly anterior to level of anterior margin of ventral sucker. Caeca approximately 4.7 times as long as wide, terminating blindly 158 from posterior end; postcaecal space representing 27% of BL.

Table II. Dimensions and ratios for species of *Isorchis* described in this study and from their original descriptions. BL = Body length; ESV = External seminal vesicle. * denotes measurement or ratio from the illustration in the original description

Species	*I. megas* sp. nov.	*I. currani* sp. nov.	*I. anomalus* sp. nov.	*I. parvus* Durio and Manter, 1969	*I. chanosi* (Zhukov, 1972)	*I. skrjabin* Ahmad, 1985
n	**13**	**6**	**9**	**9**	**7**	**17**
BL	341–406	591–695	523–709	567–912	370–510	665–1,065
Body width	130–202	192–353	232–394	262–355	210–320	370–600
Forebody	144–176	258–303	185–291	< 1/2 BL	180*	366*
Hindbody	112–154	245–294	231–297	300*	210*	342*
OS length	53–80	86–103	92–116	90*	62–71	90–140
OS width	85–117	111–141	84–137	86–128	80*	82–120
VS length	57–83	81–100	108–130	86*	75–110	75–108
VS width	56–80	69–93	107–143	86–131	79–104	75–108
Prepharynx	8–18	11–29	9–21	16*	11*	22–50
Pharynx length	39–58	50–75	46–80	48–64	41–58	42–72
Pharynx width	53–80	48–71	48–77	64–74	33–46	40–68
Oesophagus	43–69	71–132	65–94	~48–64	63*	50–62
Postcaecal space	64–113	158–224	105–157	1/4–1/5 BL	~245*	211*
Sinistral testis length	54–90	134–200	122–203	135*	100–167	110–190
Sinistral testis width	37–56	70–125	91–144	95*	41–83	68–120
Dextral testis length	42–88	118–198	127–189	128*	100–167	110–190
Dextral testis width	29–56	70–125	90–116	82*	41–83	68–120
ESV length	21–38	58–87	41–78	85*	67*	120–250
ESV width	16–36	34–56	34–99	47*	24*	31*
Hermaphroditic sac length	45–68	79–118	93–151	143*	~99*	98–160
Hermaphroditic sac width	33–46	62–84	50–134	94*	–	60–90
Internal seminal vesicle length	14–31	28–59	39–73	49*	43*	68–120
Internal seminal vesicle width	12–29	26–70	26–88	37*	22*	22–45
Gential pore from VS distance	5–13	22–47	2–11	28*	6*	92*
Ovary length	19–35	45–60	30–53	49*	42*	42–70
Ovary width	15–35	44–52	27–52	40*	37*	60–110
Vitelline follicles length	6–29	14–30	6–37	17–48*	11–20*	22–36*
Vitelline follicles width	5–20	14–28	4–28	13–32*	10–16*	13–19*
Vitellarium to anterior margin	108–146	125–185	131–183	158*	113*	277*
Vitellarium to posterior margin	31–67	47–78	62–101	88*	28*	201*
Vitelline reservoir length	23–55	35–65	29–65	–	62*	–
Vitelline reservoir width	19–70	55–83	39–75	–	56*	–
Egg number	0–1	3–12	1–2	<4	3–6	4*
Egg length	83–92	75–94	63–101	72–88	65*–79	65–77
Egg width	37–44	32–47	37–55	43–51	41–46	30–40
Distance to excretory vesicle bifurcation	46–74	115–146	119–215	–	–	226*
Width % BL	35–50	32–51	44–56	40*	61*	54*
Forebody % BL	42–49	40–46	35–41	43*	37*	46*
Hindbody % BL	32–40	40–45	39–44	45*	43*	44*
OS:VS width 1:	0.61–0.73	0.57–0.75	0.85–1.27	1	1.14*	0.90–0.91
OS: pharynx width 1:	0.58–0.73	0.39–0.60	0.46–0.63	0.60	0.52*	0.57*
Ratio of caeca width: length 1:	2.1–4.1	2.9–5.0	3.3–5.5	9.4*	8.3*	–
Postcaecal space % BL	17–28	27–36	18–25	28*	~47*	27*
Postesticular space % BL	17–36	22–37	23–30	29*	25*	29*
Hermaphroditic sac % BL	13–19	13–18	17–23	–	~20*	14*
Egg length % BL	22–26	11–15	10–17	11–12*	13–14*	9–10*
Excretory vesicle length % BL	51–70	67–76	68–77	–	–	–

Testes 2, symmetrical to slightly oblique, at approximately 3/5 of BL, elongate; sinistral testis 152 long, 96 wide; dextral testis 158 long, 96 wide. Postesticular space 32% of BL. External seminal vesicle claviform to subglobular, 77 long, 55 wide, dorsal to ventral sucker. Hermaphroditic sac 83 long, 64 wide representing 14% of BL; containing terminal genitalia; internal seminal vesicle 40 long, 39 wide; prostatic bulb elongate to subglobular; male duct short, uniting with female duct at approximately midlevel of sac; hermaphroditic duct approximately 1/2 length of hermaphroditic sac, curved; diverticula 2, uniting with hermaphroditic duct at approximately midlength of duct. Genital pore medial, 31 anterior to anterior margin of ventral sucker.

Ovary, subglobular to globular, 53 long, 51 wide, intercaecal, ventral to level of caeca, intertesticular to dorsally overlapping proximal margin of sinistral testis, partially dorsal to or posterior from ventral sucker. Laurer's canal not observed. Vitellarium follicular; follicles 14–30 long, 14–28 wide, extending anteriorly to 142 from anterior margin, extending posteriorly to 70 from posterior margin, interrupted at level of testes; vitelline reservoir transversely subglobular, 37 long, 55 wide, slightly overlapping to contiguous with posterior margin of ovary. Uterus restricted to region between posterior margin of vitelline follicles and hermaphroditic sac. Eggs 3, 85–90 long representing 14–15% of body length, 46–47 wide.

Excretory vesicle Y-shaped, bifurcating 140 from posterior margin of body, with arms extending to approximately level of midforebody, representing 67% of BL; excretory pore terminal.

Taxonomic summary

Type- and only known host: *Selenotoca multifasciata* (Richardson), spotbanded scat, (Scatophagidae).

Site of infection: Intestine.

Type-locality: Doyles boat ramp, Fannie Bay, Darwin, Northern Territory, Australia (12°26′8.7″S, 130°49′56″E); other locality: Sandy Creek, Darwin, Northern Territory, Australia (12°20′33″S, 130°53′6″E).

Specimens deposited: Holotype NMT D000516; paratypes NMT D000544, D000757 (n = 2), USNM 1254769–1254771 (n = 3).

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. KU873016 from 1 entire specimen from Doyles boat ramp and 1 entire specimen form Sandy Creek. GenBank accession no. KU873017 from 1 entire specimen from Doyles boat ramp.

Etymology: This species is named in honor of Dr Stephen Curran for his contributions to the taxonomy and systematics of haploporoid trematodes.

Remarks

Isorchis currani sp. nov. is separated from all other species of *Isorchis*, with the exception of *I. megas* sp. nov. by infecting a non-chanid host. *Isorchis currani* sp. nov. is differentiated from *I. megas* sp. nov. by its larger body size (> 550 μm; and is generally reflected in the other metrical data as well), more numerous eggs (four of our specimens had 6 or more eggs), smaller egg size relative to body length (11−15% of BL rather than >20% of BL), and more numerous vitelline follicles. Of the species of *Isorchis* from *C. chanos*, *I. currani* sp. nov. is most similar to *I. parvus* in having a similar size and shape of the body, distribution of the vitelline follicles, and length of the prepharynx; however, it can be differentiated from that species in having a genital pore that is not surrounded by large radial muscles, the ratio of the oral sucker to ventral sucker widths that is less than 1:1, and usually possessing more eggs (3–12 rather than less than 4). *Isorchis currani* sp. nov. can be further differentiated from *I. chanosi* by its its larger body size and from *I. skrjabin* by its longer oesophagus (Table II).

Isorchis anomalus sp. nov. (Figs 5–6)

Description based on 9 mature, wholemounted, unflattened specimens. Body broadly fusiform, 691 long, 350 wide at midbody representing 51% of BL. Forebody 280 long representing 41% of BL. Hindbody 281 long representing 41% of BL. Eyespot pigment lightly dispersed in anterior midforebody, more prominent dorsally. Tegumental spines 1–2 long, fine, dense, occurring over entire body surface. Oral sucker terminal, subsglobular, 106 long, 137 wide. Ventral sucker globular to subglobular, 130 long, 116 wide. Ratio of oral sucker to ventral sucker widths 1: 0.85. Prepharynx 21 long. Pharynx globular, 72 long, 73 wide. Ratio of oral sucker to pharynx width 1:0.53. Oesophagus straight to sinuous, 71 long. Intestinal bifurcation at or slightly anterior to level of anterior margin of ventral sucker. Caeca approximately 5.2 times as long as wide, terminating blindly 157 from posterior end; postcaecal space representing 23% of BL.

Testes 2, tandem or nearly so, at approximately 2/3 of BL, elongate; sinistral testis 203 long, 120 wide; dextral testis 189 long, 114 wide. Postesticular space 23% of BL. External seminal vesicle subglobular, 52 long, 91 wide, dorsal to ventral sucker. Hermaphroditic sac 151 long, 134 wide representing 22% of BL; containing terminal genitalia; internal seminal vesicle 63 long, 88 wide; prostatic bulb elongate to subglobular; male duct short, uniting with female duct at approximately midlevel of sac; hermaphroditic duct approximately 2/5 length of hermaphroditic sac; diverticula 2, uniting with hermaphroditic duct at approximately midlength of duct. Genital pore irregular, medial, 9 anterior to anterior margin of ventral sucker.

Ovary globular, 53 long, 51 wide, intercaecal, ventral to level of caeca, intertesticular to dorsally overlapping proximal margin of sinistral testis, posterior to ventral sucker. Laurer's canal not observed. Vitellarium follicular; follicles relatively numerous, 9–26 long, 11–18 wide, extending anteriorly to 131 from anterior margin, extending posteriorly to 101 from posterior margin, ventrally interrupted at level of

testes; vitelline reservoir transversely subglobular, 58 long, 75 wide, posterior to ovary. Uterus restricted to region between vitelline reservoir and hermaphroditic sac. Eggs 2, 66 long representing 10% of body length, 45 wide.

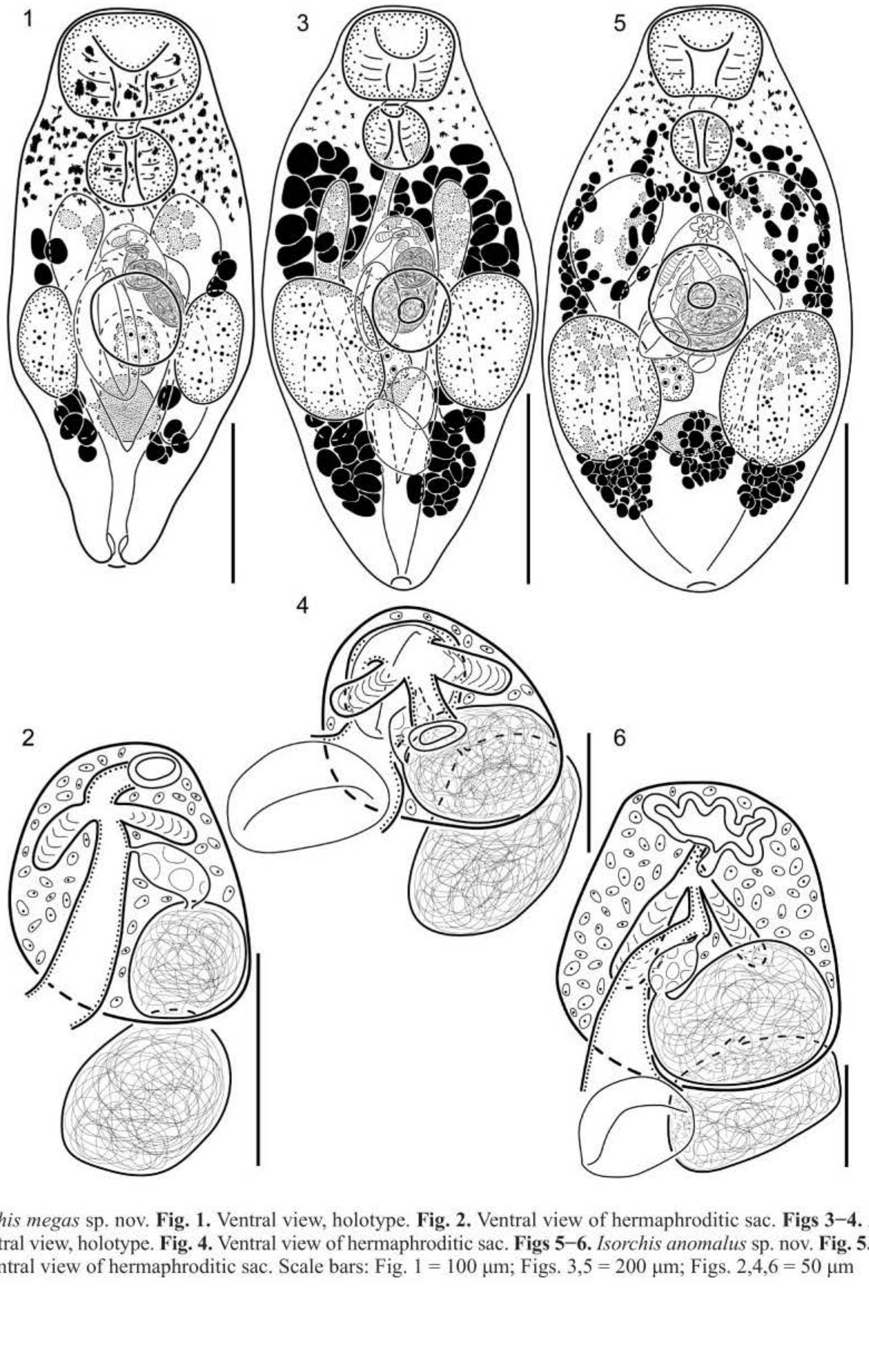
Excretory vesicle Y-shaped, bifurcating 170 from posterior margin of body, with arms extending to approximately level of midforebody, representing 74% of BL; excretory pore terminal.

Taxonomic summary

Type- and only known host: *Chanos chanos* (Forsskål), milkfish, Chanidae.

Site of infection: Intestine.

Type-locality: off Learmonth, Western Australia, Australia (22°12′41″S, 114°5′59″E).



Figs 1–2. *Isorchis megas* sp. nov. **Fig. 1.** Ventral view, holotype. **Fig. 2.** Ventral view of hermaphroditic sac. **Figs 3–4.** *Isorchis currani* sp. nov. **Fig. 3.** Ventral view, holotype. **Fig. 4.** Ventral view of hermaphroditic sac. **Figs 5–6.** *Isorchis anomalus* sp. nov. **Fig. 5.** Ventral view, holotype. **Fig. 6.** Ventral view of hermaphroditic sac. Scale bars: Fig. 1 = 100 μm; Figs. 3,5 = 200 μm; Figs. 2,4,6 = 50 μm

Specimens deposited: Holotype WAM V8553; paratypes WAM V8554–8556 (n = 3), USNM 1254772–1254775 (n = 4).

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. KU873018 from 3 entire specimens.

Etymology: The Latinised Greek, masculine "*anomalus*" meaning irregular or deviating from the normal refers to both the irregular shape of the genital pore and the fact it deviates from the typical circular to oblong genital pore shape.

Remarks

Isorchis anomalus sp. nov. is differentiated from all other species of *Isorchis* in having a genital pore that is irregular rather than one that is circular to oblong. It is most similar to *I. parvus* that was described from New Caledonia; however, *I. parvus* has larger vitelline follicles, large radial muscles surrounding the genital pore, a more fusiform body shape, and an external seminal vesicle that is nearly as long as the her-

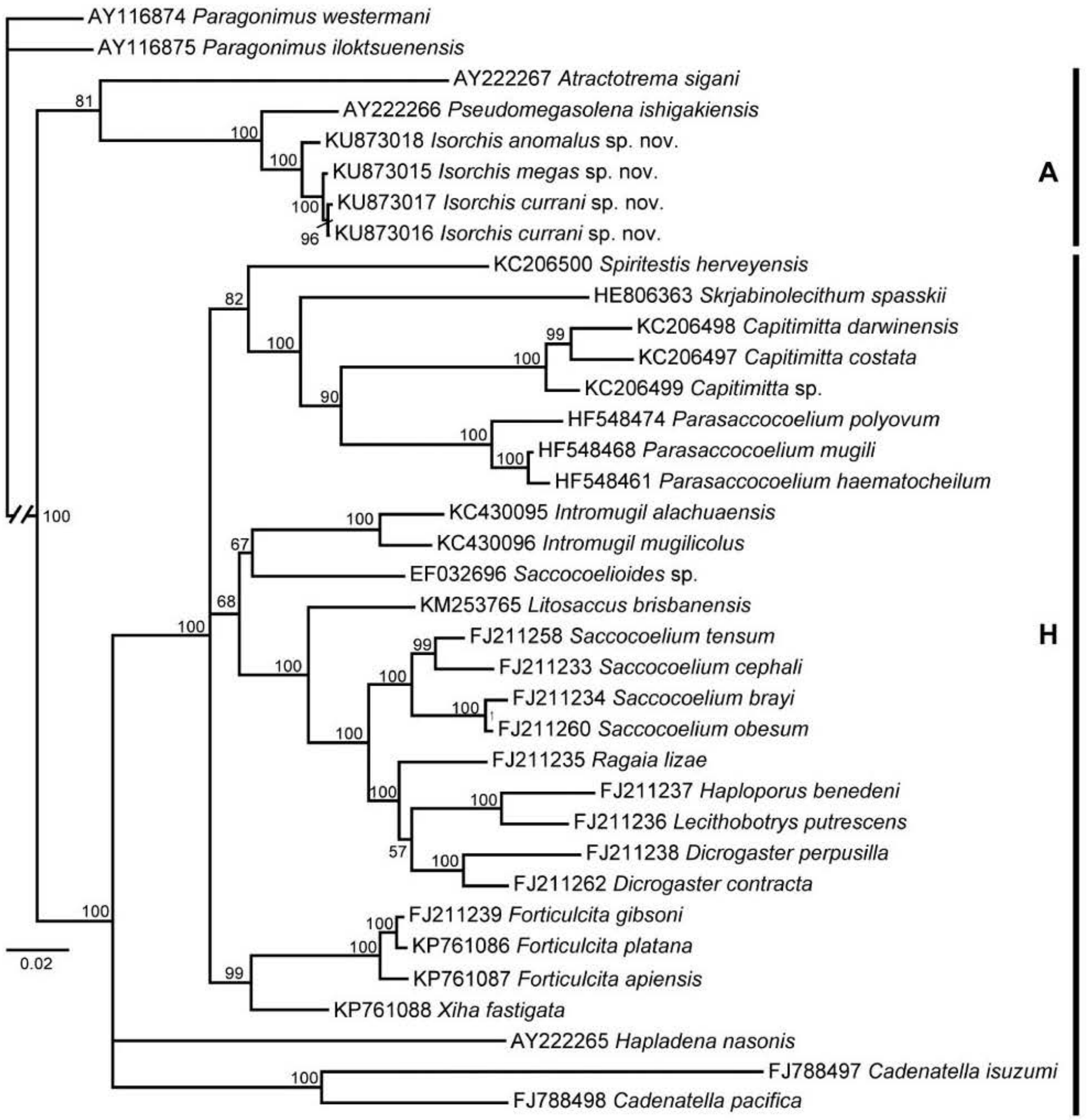


Fig 7. Phylogenetic relationships among members of the Haploporoidea resulting from Bayesian inference analysis of partial 28S rDNA sequences (GTR + I + Γ, 1,000,000 generations and a sample frequency of 100) revealing a monophyletic Atractotrematidae and *Pseudomegasolena ishigakiensis* as the sister to the three species of *Isorchis*. Vertical bars denote family. Abbreviations: A, Atractotrematidae; H, Haploporidae

Table III. Pairwise comparisons (excluding gaps) of percent nucleotide difference and number of base pair differences (in parentheses) of the ITS-1 (below the diagonal) and 28S (above the diagonal) of the three species of *Isorchis*. n = number sequenced

	n	***I. megas* sp. nov.**	***I. currani* sp. nov.**	***I. anomalus* sp. nov.**
I. megas sp. nov.	3	–	0.1–0.2 (2–3)	0.9 (13)
I. currani sp. nov.	3	0.8 (4)	–	0.9–1.0 (13–14)
I. anomalus sp. nov.	3	1.8 (9)	2.1 (11)	–

maphroditic sac rather than one that is approximately half as long as the hermaphroditic sac. *Isorchis anomalus* sp. nov. is further differentiated from *I. chanosi* in having a longer body (≥523 µm rather than ≤510 µm) and a uterus that does not extend to the posterior margin of the body. The new species is further differentiated from *I. skrjabini* Ahmad, 1985 in the more anterior extent of the vitelline field (reaching the pharynx rather than the caecal bifurcation), a shorter prepharynx (less than 30% of pharyngeal length rather than greater than 50% of pharyngeal length), a subglobular to globular ovary (rather than triangular), an excretory vesicle that extends into the forebody, and a broader body. In addition to host differences, *I. anomalus* sp. nov. can be differentiated from *I. megas* sp. nov. based on the smaller egg size relative to the body length and *I. currani* sp. nov. by a shorter postcaecal space relative to the body length (Table II).

Molecular Results

The DNA sequence fragments for the three new species of *Isorchis* encompassed a portion of the 3' end of the 18S, the ITS1, 157 bp of the 5.8S, the ITS2, and 1,393 bp of the 5' end of the 28S. No intraspecific variation was observed from sequences obtained from three specimens each of *I. megas* sp. nov. and *I. anomalus* sp. nov. Sequences obtained from two individuals of *I. currani* sp. nov. had a pyrimidine transition at position 552 in the 28S; however, no intraspecific variation was observed in the ITS1 or ITS2 sequences. The partial 18S and 5.8S rDNA sequences of all three species were identical. The sequence lengths for the ITS1 and ITS2 of *I. megas* were 514 bp and 266 bp, respectively; for *I. currani* sp. nov. were 513 bp and 266 bp, respectively; and for *I. anomalus* sp. nov. were 514 bp and 264 bp, respectively. The ITS2 sequences of *I. megas* and *I. currani* sp. nov. were identical and differed by 1.9% (5 bp) from the ITS2 sequences of *I. anomalus* sp. nov. Pairwise comparison of the ITS1 and partial 28S of the three new species are reported in Table III.

The 28S sequence alignment used for phylogenetic comparison included 2 species of *Paragonimus*, 5 atractotrematids, 2 species of *Cadenatella* Dollfus, 1946, and 26 haploporids, and it was 1,128 characters long with 638 conserved sites, 490 variable sites, and 377 informative sites. The BI analysis of partial 28S rDNA sequences (Fig. 7) used *Paragonimus westermani* Kerbert, 1878 as the outgroup based on its phylogenetic position to the Haploporoidea (Olson *et al.* 2003). The Atractotrematidae was resolved as monophyletic and sister to the Haploporidae. *Atractotrema sigani* was resolved as the sister to *Pseudomegasolena ishigaki* + the three species of *Isorchis*. The two species of *Isorchis* from the scatophagid host were each other's closest relative.

Discussion

Our study represents the first descriptions of species of *Isorchis* from Australia and a host other than *C. chanos*. Durio and Manter (1969) stated that *Isorchis* 'includes the type species, *I. parvus*, and an undescribed species collected in Australia', but did not provide information on the host or locality of the undescribed species. When making taxonomic decisions regarding species of *Isorchis*, we consider the host differences to be significant, especially because all species described to this point have been from *C. chanos*. The closer relationship of *I. megas* sp. nov. to *I. currani* sp. nov. rather than either is to *I. anomalus* sp. nov. supports this consideration and may represent a diversification in scatophagids following a host switching event from chanids; however, additional species of *Isorchis* from chanid hosts need molecular data to confirm this suggestion. Furthermore, the feeding ecologies of *C. chanos* and *S. multifasciata* are different. *Chanos chanos* is a roving herbivore and detritivore that feeds on microalgae and also on planktonic and nektonic organisms (Bagarinao 1994), whereas *Selenotoca multifasciata* is a grazer that feeds on filamentous algae, on macrophytes, and incidentally on benthic invertebrates (Lee *et al.* 1993, pers. obsv.). We also examined 12 individuals of *Scatophagus argus* (Linnaeus) from Buffalo Creek, Darwin, and did not encounter either *I. megas* sp. nov. or *I. currani* sp. nov.

Isorchis megas sp. nov. and *I. currani* sp. nov. are morphologically distinct ; *I. megas* sp. nov. is considerably smaller and has a larger egg relative to body length. We also chose to include nongravid specimens of *I. megas* sp. nov. in our description because the nongravid individuals had reproductive organs that were of similar size to those of gravid specimens and had sperm-filled seminal vesicles. Two of the specimens selected for sequencing of *I. megas* sp. nov. (one from Dampier and one from Darwin) were labeled as immature in our laboratory notebook because of the lack of eggs, but both sequences matched that of the gravid *I. megas* sp. nov. specimen. No specimen of *I. currani* sp. nov. was found from hosts collected off Dampier, but both species were collected from a pooled sample of three individuals of *S. multifasciata*, all approximately 10–12 cm long collected off Darwin. Therefore,

I. megas sp. nov. and *I. currani* sp. nov. may co-infect the same individual host, and future workers should take care when identifying non-gravid specimens of *Isorchis* from *S. multifasciata*, especially those from off Darwin. The fewer and more number of eggs relative to other species of *Isorchis* (Table III) found in *I. megas* sp. nov. and *I. currani* sp. nov., respectively, warrants further study and the collection of additional specimens of each species. *Isorchis megas* sp. nov. is particularly intriguing, as this species' eggs are approximately equal in size to those of other species of *Isorchis* (Table III) but larger related to body length. Poulin (1997) found no relationship between egg size and egg numbers after controlling for body size in his broad examination of trematode life-history traits. Therefore, the different egg allometric relationship between *I. megas* sp. nov. and *I. currani* sp. nov. may reflect different selective regimes between these two sympatric species (Poulin 2009).

The species pair of *I. megas* sp. nov. and *I. currani* sp. nov. is the third one to be reported from *S. multifasciata* off Australia. Bray (1982) described two species of *Bacciger* Nicoll, 1914 (Faustulidae Poche, 1926) from Moreton Bay, Queensland, from a host originally identified as *Mugil* sp., but Cribb *et al.* (1999) redescribed both species from *S. multifasciata* from Moreton Bay and suggested that the original host record for these species was incorrect. Pulis and Overstreet (2013) described two species of *Capitimitta* Pulis and Overstreet, 2013 (Haploporidae) from two scat species off Darwin and Cabbage Tree Creek, Queensland, and provided a sequence for yet another undescribed species of *Capitimitta* from Causeway Lake, Queensland. They found one species (*Capitimitta darwinensis* Pulis and Overstreet, 2013) only from Darwin and the other (*Capitimitta costata* Pulis and Overstreet, 2013) from off both Darwin and Cabbage Tree Creek. For both the *Bacciger* and *Capitimitta* species pairs, the major distinguishing characters are body and egg size, with the larger species also having larger eggs. *Capitimitta darwinensis* and *C. costata* are molecularly more divergent from each other (5.7%, in ITS1, 7.3% in ITS2, and 2.7% in 28S) than *I. megas* sp. nov. and *I. currani* sp. nov. are to each other (sequences for both species of *Bacciger* are not available). Interestingly, the sequences for the ITS2 of *I. megas* sp. nov. and *I. currani* sp. nov. were identical and differed by only 2–3 bp in the 28S rDNA region. However, the ITS1 sequences of *I. currani* sp. nov. were consistently 4 bp different and a single bp shorter than those of *I. megas* sp. nov. The low sequence divergence at the partial 28S region is not unexpected; however, the observed 0.1–0.2% divergence is lower than what has been reported for other haploporoid taxa (e.g., 0.8% in Blasco-Costa *et al.* 2010, 0.4% in Andres *et al.* 2015). Differences as low as a single bp in the ITS2 region of trematodes of Australian fishes have been reported (Nolan and Cribb 2006, Miller *et al.* 2009, Trieu *et al.* 2015). Identical ITS2 sequences have been reported for some putative species (see Nolan and Cribb 2005, Herrmann *et al.* 2014) that can be separated on a morphological or ecological basis.

Overstreet and Curran (2005b) considered *I. chanosi* to be 'either conspecific with *I. parvus* or very similar'. We agree that they are very similar; however, we do not consider them to be conspecific. *Isorchis chanosi* was described from the Arabian Sea off Panjim, India, whereas *I. parvus* was described off Noumea, New Caledonia. Morphologically, *I. chanosi* is distinguished from *I. parvus* by its shorter body and smaller vitelline follicles. The large geographic distance between the two species and the slight morphological differences likely indicate these are distinct species.

Ahmad (1985) stated that *Isorchis manteri* Martin, 1973 possessed characters not in common with *Isorchis*, namely an intestinal bifurcation at the level of the ventral sucker rather than anterior to it, caeca that extend further posterior, and a pretesticular uterus. Thus, he erected *Pseudisorchis* for *I. manteri* as *Pseudisorchis manteri* (Martin, 1973) Ahmad, 1985. Overstreet and Curran (2005b) agreed with this consideration, and so do we. *Pseudisorchis manteri* (and perhaps a closely related, undescribed species from *Paramugil georgii* [Ogilby, 1897] [see Overstreet and Curran 2005b]) is found in mugilid hosts, processes smaller eggs, and has larger, quincunx patterned tegumental spines (Martin 1973) rather than the minute, densely arranged tegumental spines possessed by species of *Isorchis*.

Our BI analyses showed that *Pesudomegasolena ishigakiensis* Machida and Kamiya, 1976 and species of *Isorchis* formed a clade. The close relationship of *P. ishigakiensis* and species of *Isorchis* was suggested by Overstreet and Curran (2005b) based on the presence of diverticula associated with the hermaphroditic duct of those species. Overstreet and Curran (2005b) viewed the highly host-specific nature as well as the small number of described atractotrematid species as evidence of the tentative placement of genera within the family. To help address their concern, we have doubled the described species attributed to *Isorchis*, increased the number of described atractotrematids from eight to 11 species, and supported the monophyly of the Atractotrematidae. Therefore, although the family still requires considerable attention particularly in respect to life-cycles, we concur with the placement of genera within Atractotrematidae by Overstreet and Curran (2005b). Morphologically, the Atractotrematidae is separated from the Haploporidae in processing two symmetrical or nearly symmetrical testes rather than a single or occasionally two tandem testis (as in some megasolenines). The close association of atractotrmatids with haploporids and their parasitizing herbivorous fishes may indicate that the atractotrematid life-cycle is a two host life-cycle, but this needs to be confirmed. Furthermore, we believe that the species diversity of this family is underrepresented, especially because atractotrematids seem to be restricted to the Indo-Pacific and parasitize euryhaline hosts. Cribb *et al.* (2016) considered the trematode fauna of the Indo-west Pacifc to be unevenly reported, with some areas having sustained attention whereas others (particularly French Polynesia and the Coral Triangle) being poorly known. Haploporids also parasitize euryhaline

hosts (e.g., mugilids) that are capable of serving as ecological bridges between freshwater, estuarine, and marine systems (e.g., Blasco-Costa *et al.* 2010, Pulis *et al.* 2013, Andres *et al.* 2015) that seem to promote speciation. In any event, atracotrematids appear to have not been as successful as haploporids in colonizing freshwater habitats in spite of having definitive hosts in those habitats. We hypothesize that this lack is because the atractotrematid first intermediate host will shown to be rissooid or truncatelloid snails (members of superfamilies known to serve as intermediate hosts of haploporoids) that are strictly marine.

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