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

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Abstract

Three species of *Isorhics* Durio and Manter, 1969 are described from Australian waters. *Isorhics megas* sp. nov. is described from the spotted scat, *Seleneotoca multicastrata* (Richardson), off Western Australia (WA) and Northern Territory (NT); *Isorhics currani* sp. nov. is described from *S. multifasciata* off NT; and *Isorhics anomalus* sp. nov. is described from the milkfish, *Chanos chanos* Forsskål, off WA. *Isorhics megas* sp. nov. can be differentiated from the other species of *Isorhics* 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 channid host. *Isorhics currani* sp. nov. can be differentiated from species of *Isorhics* other than *I. megas* sp. nov. by utilizing a scatophagid rather than a channid host; it is differentiated from *I. megas* sp. nov. in having eggs that are 11–15% of the body length. *Isorhics anomalus* sp. nov. can be differentiated from all other species of *Isorhics* 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 *Isorhics* and 30 other haploropoids revealed 1) the monophyly of the Atractotrematidae Yamaguti, 1939, 2) the two species of *Isorhics* infecting *S. multifasciata* were each other’s closest relative, and 3) that *Isorhics* was most closely related to *Pseudomegasolenia* Machida and Komiya, 1976 rather than *Atractotrema* Goto and Ozaki, 1929 although sequence data are not yet available for a member of *Pseudomegasolis* Ahmad, 1985.

Keywords

*Chanos*, trematode, Haploropidae, Haploropoida, Scatophagidae, phylogeny

Introduction

Jones (2005) considered the Haploropoida Nicoll, 1914 to comprise the Atractotrematidae Yamaguti, 1939 and the Haploropidae Nicoll, 1914. Members of the superfamily utilize the alimentary tract or gall bladder of marine (Atractotrematidae and Haploropidae), estuarine (Atractotrematidae and Haploropidae), and freshwater (Haploropidae) 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 hemaphroditic sac enclosing the terminal portion of the male and female reproductive structures. Olson et al. (2003) transferred both families into the superfamily Gorgoderoidae 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 Haploropoida.

Durio and Manter (1969) and Ahmad (1985) considered the Atractotrematidae a junior synonym of the Haploropidae, 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 Haploropidae.

1976. *Isorchis* is differentiated from *Atractotremata* in having testes located in the hindbody; from *Pseudosorhics* in having the ovary at the level of the testes; and from *Pseudomesogenselena* in having a fusiform rather than circular body shape as well as parasitizing chinid 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 *Krusadairema* Zhukov, 1972 for *Krusadairema chanosi* Zhukov, 1972 collected from *C. chanos* off Krusadai Island, India. Ahmad (1985) described *Isorchis skjabinii* Ahmad, 1985 from *C. chanos* from the Arabian Sea off Goa, India. He also considered *Krusadairema* a junior synonym of *Isorchis* and transferred *Krusadairema 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 Atractotrematae.

**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, 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 n-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'-CCGCGTGCTACTACCGATTG-3') and reverse primer IN150R (5'-GCTATCCTGAAGGAAAACCTCG-3'). These PCR primers and multiple internal primers were used in sequencing reactions. The internal forward primers were DIGL2 (5'-AACCATATCACTAAGCGG-3'), 300F (5'-CAAGTACCGTGAGGGAAAGTTG-3'), and 900F (5'-CGGTTGAGGAAACGACCAA-3') and the internal reverse primers were 300R (5'-CAACCTT CCTCCACGGTGATCTTG-3'), DIGL2R (5'-CCGGTATGTGATACTCG-3'), and ECD2 (5'-CTTTGGTCGGTGTTTCAAGACGCGG-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-sequence 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 Sequencer™ (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 6.61b (Katoh et al. 2005) with 1,000 cycles of iterative refinement and the genepair 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 3' and 5' 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 + T). 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 (burnin = 2,500), and nodal support was estimated by posterior probabilities.
Table I. Sequences from GenBank used for phylogenetic analysis in this study

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Host</th>
<th>GenBank</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Cadenatellinae’</td>
<td><em>Cadenatellina izumi</em> Machida, 1993</td>
<td><em>Kyphosus vaigiensis</em> Quoy and Gaimard</td>
<td>FJ788497</td>
<td>Bray et al. (2009)</td>
</tr>
<tr>
<td></td>
<td><em>Cadenatellina pacifica</em> (Yamaguti, 1970)</td>
<td><em>Kyphosus vaigiensis</em></td>
<td>FJ788498</td>
<td>Bray et al. (2009)</td>
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<td>Haploporidae</td>
<td><em>Hapladena nasonis</em> Yamaguti, 1970</td>
<td><em>Naso unicornis</em> (Forsskal)</td>
<td>AY222265</td>
<td>Olson et al. (2003)</td>
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<td><em>Dicrogaster contracta</em> Looss, 1902</td>
<td><em>Liza aurata</em> (Risso)</td>
<td>FJ211261</td>
<td>Blasco-Costa et al. (2009a)</td>
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<td></td>
<td><em>Dicrogaster perpusilla</em> Looss, 1902</td>
<td><em>Liza ramada</em> (Risso)</td>
<td>FJ211238</td>
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<td><em>Haploporus benedeni</em> (Stossich, 1887)</td>
<td><em>Liza ramada</em></td>
<td>FJ211237</td>
<td>Blasco-Costa et al. (2009a)</td>
</tr>
<tr>
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<td><em>Litosaccus brisbanensis</em> Martin, 1974</td>
<td><em>Liza saliens</em> (Risso)</td>
<td>FJ211236</td>
<td>Blasco-Costa et al. (2009a)</td>
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<td><em>Lecithobotylus putrescens</em> Looss, 1902</td>
<td><em>Liza saliens</em> (Risso)</td>
<td>FJ211234</td>
<td>Blasco-Costa et al. (2009a)</td>
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<td></td>
<td><em>Saccocoeilium brayi</em> Blasco-Costa, Balbuena, Raga, Kostadinova, and Olson, 2010</td>
<td><em>Mugil cephalus</em></td>
<td>FJ211233</td>
<td>Blasco-Costa et al. (2009a)</td>
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<td><em>Saccocoeilium obsesus</em> Looss, 1902</td>
<td><em>Liza ramada</em></td>
<td>FJ211259</td>
<td>Blasco-Costa et al. (2009a)</td>
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<td><em>Saccocoeilium tensum</em> Looss, 1902</td>
<td><em>Liza aurata</em></td>
<td>FJ211258</td>
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<td><em>Saccocoeilioides</em> sp.</td>
<td><em>Poeciliidae</em> sp.</td>
<td>EF022696</td>
<td>Curran et al. (2006)</td>
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<td><em>Capitimitta costata</em> Pulis and Overstreet, 2013</td>
<td><em>Selenotoca multifasciata</em> (Richardson)</td>
<td>KC206497</td>
<td>Pulis and Overstreet (2013)</td>
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<td><em>Capitimitta darwinesis</em> Pulis and Overstreet, 2013</td>
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<td><em>Solenotoca multifasciata</em></td>
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<td>Pulis and Overstreet (2013)</td>
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<td><em>Spiritesris herveyensis</em> Pulis and Overstreet, 2013</td>
<td><em>Moolgarda sebeli</em> (Forsskål)</td>
<td>KC206500</td>
<td>Pulis and Overstreet (2013)</td>
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<td><em>Intromugil alachuaensis</em> Pulis, Fayton, Curran and Overstreet, 2013</td>
<td><em>Mugil cephalus</em></td>
<td>KC430095</td>
<td>Pulis et al. (2013)</td>
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<tr>
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<td><em>Intromugil mugilicolus</em> (Shirman, 1964)</td>
<td><em>Mugil cephalus</em></td>
<td>KC430096</td>
<td>Pulis et al. (2013)</td>
</tr>
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<td><em>Parasaccocoeilium haematocheilum</em> Besprozvannykh, Atopkin, Ermolenko and Nikitenko, 2014</td>
<td><em>Liza haematocheilum</em> (Temminck and Schlegel)</td>
<td>HF548461</td>
<td>Besprozvannykh et al. (2014)</td>
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<td><em>Parasaccocoeilium mugili</em> Zhukov, 1971</td>
<td><em>Liza haematocheilum</em></td>
<td>HF548468</td>
<td>Besprozvannykh et al. (2014)</td>
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<td><em>Parasaccocoeilium polyovum</em> Besprozvannykh, Atopkin, Ermolenko, and Nikitenko, 2014</td>
<td><em>Liza haematocheilum</em></td>
<td>HF548474</td>
<td>Besprozvannykh et al. (2014)</td>
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<td></td>
<td><em>Xia fastigata</em> (Thatcher and Sparks, 1958)</td>
<td><em>Mugil cephalus</em> Linnaeus</td>
<td>KP761088</td>
<td>Andres et al. (2015)</td>
</tr>
<tr>
<td>Paragonimidae</td>
<td><em>Paragonimus westermani</em> (Kerber, 1878)</td>
<td><em>Canis lupus familiaris</em> Linnaeus</td>
<td>AY116874</td>
<td>Olson et al. (2003)</td>
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<td><em>Paragonimus lokiiscuenensis</em> Chen, 1940</td>
<td><em>Rattus norvegicus</em> (Berkenhout, 1769)</td>
<td>AY116875</td>
<td>Olson et al. (2003)</td>
</tr>
</tbody>
</table>
(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 non-gravid (all from off
Dampier), wholemounted, unflattened specimens. Body fusiform,
tapering posteriorly, 348 long, 147 wide at midbody represent-
ing 42% of body length (BL). Forebody 163 long represent-
ing 47% of BL. Hindbody 128 long representing 37% of
BL. Eyespot pigment primarily dispersed in forebody (dis-
persed over anterior 3/5 of BL in 1 specimen), more promi-
inent dorsally. Tegumental spines ≤1 long, fine, occurring over
entire body surface. Oral sucker terminal, transversely sub-
globular, 67 long, 85 wide. Ventral sucker sub-globular,
57 long, 59 wide. Ratio of oral sucker width to ventral sucker
length 1: 0.69. Prepharynx 11 long. Pharynx transversely sub-
globular, 43 long, 56 wide. Ratio of oral sucker to pharynx
length 1:0.66. Oesophagus straight to sinuous, 55 long. In-
testinal 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; sinis-
tral testis 69 long, 38 wide; dextral testis 70 long, 37 wide.
Posttesticular space 32% of BL. External seminal vesicle clav-
iform 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, interca-
ecral, 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 ante-
riorly 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 con-
tiguous with posterior margin of ovary. Uterus restricted to re-
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 seat. (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; paraatypes
WAM V8550–8552 (n = 3), NTM D000769, D000772,
D001328, D001567 (n = 4), USNM 1254765–1254768 (n = 4).

Representative DNA sequences: Partial l 8S, entire ITS re-

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 distribu-
tion of vitelline follicles. Isorchis megas is further separated
from I. parvus and I. skrjabin in having an intestinal bifura-
tion 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 44% of BL. Hindbody 260 long representing 44%
of BL. Eyespot pigment lightly dispersed in forebody (dis-
sored more so in anterior region, partial (D1–D3) 28S: GenBank accession no. KU873015
from 2 entire specimens from Dampier and 1 entire specimen
from Darwin.

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specimens. Body fusiform, 591 long, 251 wide at midbody
representing 44% of BL. Hindbody 260 long representing 44%
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 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.

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.
<table>
<thead>
<tr>
<th>Species</th>
<th>I. megas sp. nov.</th>
<th>I. carrani sp. nov.</th>
<th>I. anomalus sp. nov.</th>
<th>I. parvus Durio and Mant, 1969</th>
<th>I. chausi Zhukov, 1972</th>
<th>I. skrabina Ahmad, 1985</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>13</td>
<td>6</td>
<td>9</td>
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<tr>
<td>BL</td>
<td>341-406</td>
<td>591-695</td>
<td>523-709</td>
<td>567-912</td>
<td>379-510</td>
<td>665-1065</td>
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<td>Body width</td>
<td>130-202</td>
<td>192-353</td>
<td>232-394</td>
<td>262-355</td>
<td>210-320</td>
<td>370-600</td>
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<td>Forebody</td>
<td>144-176</td>
<td>258-303</td>
<td>185-291</td>
<td>&lt;1/2 BL</td>
<td>180*</td>
<td>366*</td>
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<td>Hindbody</td>
<td>112-154</td>
<td>245-294</td>
<td>231-297</td>
<td>300*</td>
<td>210*</td>
<td>342*</td>
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<td>OS length</td>
<td>53-80</td>
<td>86-103</td>
<td>92-116</td>
<td>90*</td>
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<td>111-141</td>
<td>84-137</td>
<td>86-128</td>
<td>80*</td>
<td>82-120</td>
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<tr>
<td>VS length</td>
<td>57-83</td>
<td>81-100</td>
<td>108-130</td>
<td>86*</td>
<td>75-110</td>
<td>75-108</td>
</tr>
<tr>
<td>VS width</td>
<td>56-80</td>
<td>69-93</td>
<td>107-143</td>
<td>86-131</td>
<td>79-104</td>
<td>75-108</td>
</tr>
<tr>
<td>Prepharynx</td>
<td>8-18</td>
<td>11-29</td>
<td>9-21</td>
<td>16*</td>
<td>11*</td>
<td>22-50</td>
</tr>
<tr>
<td>Pharynx length</td>
<td>39-58</td>
<td>50-75</td>
<td>46-80</td>
<td>48-64</td>
<td>41-58</td>
<td>42-72</td>
</tr>
<tr>
<td>Pharynx width</td>
<td>53-80</td>
<td>48-71</td>
<td>48-77</td>
<td>64-74</td>
<td>33-46</td>
<td>40-68</td>
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<tr>
<td>Oesophagus</td>
<td>43-69</td>
<td>71-132</td>
<td>65-94</td>
<td>-48-64</td>
<td>63*</td>
<td>50-62</td>
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<tr>
<td>Postcaecal space</td>
<td>64-113</td>
<td>158-224</td>
<td>105-157</td>
<td>14-1/5 BL</td>
<td>-245*</td>
<td>211*</td>
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<tr>
<td>Sinistral testis length</td>
<td>54-90</td>
<td>134-200</td>
<td>122-203</td>
<td>135*</td>
<td>100-167</td>
<td>110-190</td>
</tr>
<tr>
<td>Sinistral testis width</td>
<td>37-56</td>
<td>70-125</td>
<td>91-144</td>
<td>95*</td>
<td>41-83</td>
<td>68-120</td>
</tr>
<tr>
<td>Dextral testis length</td>
<td>42-88</td>
<td>118-198</td>
<td>127-189</td>
<td>128*</td>
<td>100-167</td>
<td>110-190</td>
</tr>
<tr>
<td>Dextral testis width</td>
<td>29-56</td>
<td>70-125</td>
<td>90-116</td>
<td>82*</td>
<td>41-83</td>
<td>68-120</td>
</tr>
<tr>
<td>ESV length</td>
<td>21-38</td>
<td>58-87</td>
<td>41-78</td>
<td>85*</td>
<td>67*</td>
<td>120-250</td>
</tr>
<tr>
<td>ESV width</td>
<td>16-36</td>
<td>35-64</td>
<td>35-99</td>
<td>47*</td>
<td>24*</td>
<td>31*</td>
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<tr>
<td>Hermaphroditic sac length</td>
<td>45-68</td>
<td>79-118</td>
<td>93-151</td>
<td>143*</td>
<td>~99*</td>
<td>98-160</td>
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<tr>
<td>Hermaphroditic sac width</td>
<td>33-46</td>
<td>62-84</td>
<td>50-134</td>
<td>94*</td>
<td>~60</td>
<td>90-90</td>
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<tr>
<td>Internal seminal vesicle length</td>
<td>14-31</td>
<td>28-59</td>
<td>39-73</td>
<td>49*</td>
<td>43*</td>
<td>68-120</td>
</tr>
<tr>
<td>Internal seminal vesicle width</td>
<td>12-29</td>
<td>26-70</td>
<td>26-88</td>
<td>37*</td>
<td>22*</td>
<td>22-45</td>
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<tr>
<td>Gential pore from VS distance</td>
<td>5-13</td>
<td>22-47</td>
<td>2-11</td>
<td>28*</td>
<td>6*</td>
<td>92*</td>
</tr>
<tr>
<td>Ovary length</td>
<td>19-35</td>
<td>45-60</td>
<td>30-53</td>
<td>49*</td>
<td>42*</td>
<td>42-70</td>
</tr>
<tr>
<td>Ovary width</td>
<td>15-35</td>
<td>44-52</td>
<td>27-52</td>
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<td>37*</td>
<td>60-110</td>
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<tr>
<td>Vitelline follicles length</td>
<td>6-29</td>
<td>14-30</td>
<td>6-37</td>
<td>17-48*</td>
<td>11-20*</td>
<td>22-36*</td>
</tr>
<tr>
<td>Vitelline follicles width</td>
<td>5-20</td>
<td>14-28</td>
<td>4-28</td>
<td>13-32*</td>
<td>10-16*</td>
<td>13-19*</td>
</tr>
<tr>
<td>Vitellarium to anterior margin</td>
<td>108-146</td>
<td>125-185</td>
<td>131-183</td>
<td>158*</td>
<td>113*</td>
<td>277*</td>
</tr>
<tr>
<td>Vitellarium to posterior margin</td>
<td>31-67</td>
<td>47-78</td>
<td>62-101</td>
<td>88*</td>
<td>28*</td>
<td>201*</td>
</tr>
<tr>
<td>Vitelline reservoir length</td>
<td>23-55</td>
<td>35-65</td>
<td>29-65</td>
<td>-</td>
<td>62*</td>
<td>-</td>
</tr>
<tr>
<td>Vitelline reservoir width</td>
<td>19-70</td>
<td>55-83</td>
<td>39-75</td>
<td>-</td>
<td>56*</td>
<td>-</td>
</tr>
<tr>
<td>Egg number</td>
<td>0-1</td>
<td>3-12</td>
<td>1-2</td>
<td>&lt;4</td>
<td>3-6</td>
<td>4*</td>
</tr>
<tr>
<td>Egg length</td>
<td>83-92</td>
<td>75-94</td>
<td>63-101</td>
<td>72-88</td>
<td>65-79</td>
<td>65-77</td>
</tr>
<tr>
<td>Egg width</td>
<td>37-44</td>
<td>32-47</td>
<td>37-55</td>
<td>43-51</td>
<td>41-46</td>
<td>30-40</td>
</tr>
<tr>
<td>Distance to excretoey vesicle bifurcation</td>
<td>46-74</td>
<td>115-146</td>
<td>119-215</td>
<td>-</td>
<td>-</td>
<td>226*</td>
</tr>
<tr>
<td>Width % BL</td>
<td>35-50</td>
<td>32-51</td>
<td>44-56</td>
<td>40*</td>
<td>61*</td>
<td>54*</td>
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<tr>
<td>Forebody % BL</td>
<td>42-49</td>
<td>40-46</td>
<td>35-41</td>
<td>43*</td>
<td>37*</td>
<td>46*</td>
</tr>
<tr>
<td>Hindbody % BL</td>
<td>32-40</td>
<td>40-45</td>
<td>39-44</td>
<td>45*</td>
<td>43*</td>
<td>44*</td>
</tr>
<tr>
<td>OS:VS width 1:</td>
<td>0.61-0.73</td>
<td>0.57-0.75</td>
<td>0.85-1.27</td>
<td>1</td>
<td>1.14*</td>
<td>0.90-0.91</td>
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<tr>
<td>OS:pharynx width 1:</td>
<td>0.58-0.73</td>
<td>0.39-0.60</td>
<td>0.46-0.63</td>
<td>0.60</td>
<td>0.52*</td>
<td>0.57*</td>
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<tr>
<td>Ratio of caecum width: length 1:</td>
<td>2.1-4.1</td>
<td>2.9-5.0</td>
<td>3.3-5.5</td>
<td>9.4*</td>
<td>8.3*</td>
<td>-</td>
</tr>
<tr>
<td>Postcecal space % BL</td>
<td>17-28</td>
<td>27-36</td>
<td>18-25</td>
<td>28*</td>
<td>-47*</td>
<td>27*</td>
</tr>
<tr>
<td>Postesticular space % BL</td>
<td>17-36</td>
<td>22-37</td>
<td>23-30</td>
<td>29*</td>
<td>25*</td>
<td>29*</td>
</tr>
<tr>
<td>Hermaphroditic sac % BL</td>
<td>13-19</td>
<td>13-18</td>
<td>17-23</td>
<td>-</td>
<td>20*</td>
<td>14*</td>
</tr>
<tr>
<td>Egg length % BL</td>
<td>22-26</td>
<td>11-15</td>
<td>10-17</td>
<td>11-12*</td>
<td>13-14*</td>
<td>9-10*</td>
</tr>
<tr>
<td>Excretory vesicle length % BL</td>
<td>51-70</td>
<td>67-76</td>
<td>68-77</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
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, interaccael, 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, eyespot pigment lightly dispersed in anterior midforebody, extending 41% of BL. Hindbody 281 long representing 41% of BL. Eyespot pigment lightly dispersed (Table II).

Remarks

I. currani sp. nov. is separated from all other species of Isorchis, with the exception of I. megalus sp. nov. by infecting a non-chondid host. I. currani sp. nov. is differentiated from I. megalus 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). I. currani sp. nov. can be further differentiated from I. chanosi by its larger body size and from I. skrjabini 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. Segmentation spines 1–2 long, fine, dense, occurring over entire body surface. Oral sucker terminal, subglobular, 106 long, 137 wide. Ventral sucker globular to subglobular, 130 long, 116 wide. Ratio of oral sucker to ventral sucker widths 1: 0.85. Pharynx globular, 72 long, 73 wide. Ratio of oral sucker to pharynx width 1: 0.53. Oesophagus straight to sinuous, 71 long. Intestinal bifurcation or at slightly anterior to level of posterior margin of ventral sucker. Caeca approximately 5.2 times as long as wide, terminating blindly 157 from posterior end; postaccael space representing 23% of BL.

Ovary globular, 53 long, 51 wide, interaccael, ventral to level of caeca, intertesticular to dorsally overlapping proximal margin of sinistral testis, posterior to ventral sucker. Lauser'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.
Description of *Isorchis* from Australia

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-

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**Fig 7.** Phylogenetic relationships among members of the Haploporoidea resulting from Bayesian inference analysis of partial 28S rDNA sequences (GTR + I + F, 1,000,000 generations and a sample frequency of 100) revealing a monophyletic Atractotrematidae and *Pseudomegasolenia ishigakiensis* as the sister to the three species of *Isorchis*. Vertical bars denote family. Abbreviations: A, Atractotrematidae; H, Haploporidae.
maphroditic sac rather than one that is approximately half as long as the hermaproditic 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 prophyarynx (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 Haploporidea (Olson et al. 2003). The Atractotrematidae was resolved as monophyletic

<table>
<thead>
<tr>
<th></th>
<th>I. megas sp. nov.</th>
<th>I. currani sp. nov.</th>
<th>I. anomalus sp. nov.</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>3</td>
<td>0.8 (4)</td>
<td>1.8 (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1–0.2 (2–3)</td>
<td>0.9 (13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.9–1.0 (13–14)</td>
<td>-</td>
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</table>

**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 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 riving 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 Scaphognathus 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. Braty (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) described 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 m mogul 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 Pesudomegaso!ena 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 atracotrmatid 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 atracotrmatids 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 testes (as in some megasolenines). The close association of atracotrmatids with haploporids and their parasitizing herbivorous fishes may indicate that the atracotrmatid 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 atracotrmatids seem to be restricted to the Indo-Pacific and parasite euryhaline hosts. Cribb et al. (2016) considered the trematode fauna of the Indo-west Pacific to be unevenly reported, with some areas having sustained attention whereas others (particularly French Polynesia and the Coral Triangle) being poorly known. Haploporids also parasite 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 atracotrematid first intermediate host will shown to be rissooid or truncatellid snails (members of superfamilies known to serve as intermediate hosts of haploporoids) that are strictly marine.

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