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Erection of the Haploporid Genus *Litosaccus* n. g. and Its Phylogenetic Relationship within the Haploporidae Nicoll, 1914

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Abstract

Litosaccus n. g. is erected for *Paralecithobotrys brisbanensis* Martin, 1974 n. comb. for which an amended description is given. The new genus is morphologically similar to the haploporine *Lecithobotrys* Looss, 1902 but with a more elongate and cylindrical body; an infundibuliform oral sucker; a thin-walled hermaphroditic sac; a shallow genital atrium; and unequal, cylindrical, and elongated caeca. It also resembles *Pseudolecithobotrys* Blasco-Costa, Gibson, Balbuena, Raga & Kostadinova, 2009, but the only member of that genus has a hermaphroditic sac that is twice the length of the ventral sucker, a hermaphroditic duct with intensely staining cuboidal cells, an elongate testis, and single or paired caeca. A Bayesian inference analysis of partial 28S rDNA sequences of *L. brisbanensis* and 24 other haploporoids revealed that *L. brisbanensis* grouped with other haploporines and placed *Intromugil* Overstreet & Curran, 2005 in a clade with the chalcinotrematine *Saccocoelioides* Szidat, 1954 rather than the other seven tested waretrematine species. This analysis represents the first phylogenetic study of the Haploporidae Nicoll, 1914 that incorporates a haploporine from outside of the Mediterranean Sea.

Introduction

Martin (1974) described the haploporid *Paralecithobotrys brisbanensis* Martin, 1974 from the Brisbane River, Queensland (QLD), Australia, in *Mugil cephalus* Linnaeus. In a review of the Haploporidae Nicoll, 1914, Overstreet & Curran (2005) reported that the holotype of *P. brisbanensis* had been temporarily lost, but they examined specimens of *P. brisbanensis* collected by RMO from the type-host, near the type-locality. They transferred *P. brisbanensis* to *Lecithobotrys* Looss, 1902 as *Lecithobotrys brisbanensis* (Martin, 1974) Overstreet & Curran, 2005 because members of *Paralecithobotrys* Teixeira de Freitas, 1947 have vitelline follicles distributed in a patchy manner rather than in two distinct, grape-like clusters (as in *Lecithobotrys*) and are found in non-mugilid, freshwater fishes in South America and Africa. Additionally, they considered *Paralecithobotrys* to belong in the subfamily Chalcinotrematinae Overstreet & Curran, 2005. Blasco-Costa et al. (2009b) revised *Haploporus* Looss, 1902 and *Lecithobotrys* and considered *L. brisbanensis* to be a *species inquirenda*. They considered it to possess morphological features inconsistent with *Lecithobotrys*, namely, an elongate cylindrical body, a weakly muscularized genital atrium, a poorly developed hermaphroditic sac, and an armed hermaphroditic duct. Citing the loss of the type-material and morphological differences between *Lecithobotrys* and *L. brisbanensis* sp. inq., Blasco-Costa et al. (2009b) suggested that description of new material from the type-host and type-locality was needed to assess the generic affiliation of *L. brisbanensis*.

Blasco-Costa et al. (2009a) provided the first molecular phylogenetic hypothesis for the Haploporidae based on sequences of partial 28S ribosomal DNA (rDNA), and it included the type-species of *Lecithobotrys*, *Lecithobotrys putrescens* Looss, 1902, and eight other haploporine genera. Since then, four additional works on haploporids have incorporated molecular data. Pulis & Overstreet (2013) generated the second molecular hypothesis for the family and included four waretrematines. Pulis et al. (2013) described *Intromugil alachuaensis* Pulis, Fayton, Curran & Overstreet, 2013 and provided sequences of the internal transcribed spacer region (ITS1-5.8S-ITS2) and partial 28S rDNA for two species of *Intromugil* Overstreet & Curran, 2005. Besprozvannykh et al. (2014) restored *Parasaccocoelium* Zhukov, 1971 and resolved three species of that genus close to the waretrematine genus *Capitimitta* Pulis & Overstreet, 2013 based on analysis of partial 28S rDNA sequence data. Bray et al. (2014) used the same gene region to demonstrate that *Cadenatella* Dollfus, 1946 belongs within the superfamily Haploporoidea Nicoll, 1914, despite the absence of a hermaphroditic sac in its members, for which they used subfamily name Cadenatellinae Gibson & Bray, 1982. Here we report on freshly collected specimens of *L. brisbanensis* from the type-host near the type-locality, provide supplemental material, and present a Bayesian inference (BI) analysis of partial 28S rDNA sequences to test its phylogenetic placement within the Haploporidae.

Materials and methods

During March, 2010 three moribund specimens resembling *L. brisbanensis* sp. inq. were collected from *M. cephalus* cast-netted off Shorncliffe, Queensland (QLD), Australia, following the method of Cribb & 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; then most of the saline was decanted, and the worms were killed by pouring hot (not boiling) water over them and then fixed in 70% ethanol. Additional specimens of *L. brisbanensis* sp. inq. were collected from *M. cephalus* during: April 1984 off Redland Bay, QLD; January 1995 from the Brisbane River, Toowong, QLD; and November 1997 from off Shorncliffe and Wynnum Creek, QLD. Worms were stained in Mayer's haematoxylin or Van Cleave's haematoxylin, dehydrated in a graded ethanol series, cleared in clove oil (Van Cleave's) or methyl salicylate (Mayer's), and mounted permanently in Canada balsam (Van Cleave's) or Damar gum (Mayer's). Measurements were made using a compound microscope equipped with a differential interference contrast, a Cannon EOS Rebel T1i camera, and calibrated digital software (iSolutions Lite ©). All measurements are in micrometers, and data for the illustrated specimen are followed by the range of data for the other specimens in parentheses. Terminology of the hermaphroditic sac and its structures follows the terms used by Pulis & Overstreet (2013).

Genomic DNA was isolated from two entire specimens using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the instructions provided. DNA fragments c. 2,550 base pairs (bp) long, comprising the 3′ end of the 18S nuclear rRNA gene, internal transcribed spacer region (including ITS1 + 5.8S + ITS2) 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′-CGC CCG TCG CTA CCG ATT G-3′) and reverse primer 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′). These PCR primers and multiple internal primers were used in sequencing reactions. The internal forward primers were DIGL2 (5′-AAG CAT ATC ACT AAG CGG-3′), 300F (5′-CAA GTA CCG TGA GGG AAA GTT G-3′), and 900F (5′-CCG TCT TGA AAC ACG GAC CAA G-30) and the internal reverse primers were 300R (5′-CAA CTT TCC CTC ACG GTA CTT G-3′), DIGL2R (5′-CCG CTT AGT GAT ATG CTT-3′), and ECD2 (5′-CTT GGT CCG TGT TTC AAG ACG GG-3′). The resulting PCR products were excised from PCR gels using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, California, USA) following the manufacturer's 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 (Gene-Codes Corp., Ann Arbor, Michigan, USA, Version 4.10.1) and submitted to GenBank. Sequences of related species were obtained from GenBank (Table 1). 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 alignment utilized two atractotrematids, two species of *Cadenatella*, and 22 haploporids with the paragonimid *Paragonimus westermani* (Kerbert, 1878) as the outgroup based on its phylogenetic position relative to the Haploporoidea (Olson et al., 2003). Phylogenetic analysis of the data was performed using BI with MrBayes 3.1.2 software (Huelsenbeck & 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: nst = 6, rates = invgamma, ngen = 1,000,000, and samplefreq = 100. Burn-in value was 1,500 estimated by plotting the log-probabilities against generation and visualizing plateau in parameter values (sump burnin = 1,500), and nodal support was estimated by posterior probabilities (sumt) (Huelsenbeck et al., 2001) with all other settings left as default.

Table 1. Sequences used for phylogenetic analysis in this study

Family	Species	Host	GenBank Accession No.	Reference
Paragonimidae	*Paragonimus westermani* (Kerber, 1878)	*Canis lupus familiaris* Linnaeus	AY116874	Olson et al. (2003)
Atractotrematidae	*Atractotrema sigani* Durio & Manter, 1969	*Siganus lineatus* (Valenciennes)	AY222267	Olson et al. (2003)
Atractotrematidae	*Pseudomegasolena ishigakiense* Machida & Kamiya, 1976	*Scarus rivulatus* Valenciennes	AY222266	Olson et al. (2003)
"Cadenatellinae"	*Cadenatella isuzumi* Machida, 1993	*Kyphosus vaigiensis* Quoy & Gaimard	FJ788497	Bray et al. (2009)
"Cadenatellinae"	*Cadenatella pacifica* (Yamaguti, 1970)	*Kyphosus vaigiensis* Quoy & Gaimard	FJ788498	Bray et al. (2009)
Haploporidae	*Hapladena nasonis* Yamaguti, 1970	*Naso unicornis* (Forsskål)	AY222265	Olson et al. (2003)
Haploporidae	*Dicrogaster contracta* Looss, 1902	*Liza aurata* (Risso)	FJ211261	Blasco-Costa et al. (2009a)
Haploporidae	*Dicrogaster perpusilla* Looss, 1902	*Liza ramada* (Risso)	FJ211238	Blasco-Costa et al. (2009a)
Haploporidae	*Forticulcita gibsoni* Blasco-Costa, Montero, Balbuena, Raga & Kostadinova, 2009	*Mugil cephalus* Linnaeus	FJ211239	Blasco-Costa et al. (2009a)
Haploporidae	*Haploporus benedeni* (Stossich, 1887)	*Liza ramada* (Risso)	FJ211237	Blasco-Costa et al. (2009a)
Haploporidae	*Lecithobotrys putrescens* Looss, 1902	*Liza saliens* (Risso)	FJ211236	Blasco-Costa et al. (2009a)
Haploporidae	*Ragaia lizae* Blasco-Costa, Montero, Gibson, Balbuena & Kostadinova, 2009	*Liza aurata* (Risso)	FJ211235	Blasco-Costa et al. (2009a)
Haploporidae	*Saccocoelium brayi* Blasco-Costa, Balbuena, Raga, Kostadinova & Olson, 2010	*Liza saliens* (Risso)	FJ211234	Blasco-Costa et al. (2009a)
Haploporidae	*Saccocoelium cephalic* Blasco-Costa, Montero, Gibson, Balbuena, Raga & Kostadinova, 2009	*Mugil cephalus* Linnaeus	FJ211233	Blasco-Costa et al. (2009a)

Table 1. *Continued*

Haploporidae	*Saccocoelium obesum* Looss, 1902	*Liza ramada* (Risso)	FJ211259	Blasco-Costa et al. (2009a)
Haploporidae	*Saccocoelium tensum* Looss, 1902	*Liza aurata* (Risso)	FJ211258	Blasco-Costa et al. (2009a)
Haploporidae	*Saccocoelioides* sp.	Poeciliidae Garman	EF032696	Curran et al. (2006)
Haploporidae	*Capitimitta costata* Pulis & Overstreet, 2013	*Selenotoca multifasciata* (Richardson)	KC206497	Pulis & Overstreet (2013)
Haploporidae	*Capitimitta darwinensis* Pulis & Overstreet, 2013	*Selenotoca multifasciata* (Richardson)	KC206498	Pulis & Overstreet (2013)
Haploporidae	*Capitimitta* sp.	*Selenotoca multifasciata* (Richardson)	KC206499	Pulis & Overstreet (2013)
Haploporidae	*Spiritestis herveyensis* Pulis & Overstreet, 2013	*Moolgarda seheli* (Forsskål)	KC206500	Pulis & Overstreet (2013)
Haploporidae	*Intromugil alachuaensis* (Shireman, 1964)	*Mugil cephalus* Linnaeus	KC430095	Pulis et al. (2013)
Haploporidae	*Intromugil mugilicolus* Pulis, Fayton, Curran & Overstreet, 2013	*Mugil cephalus* Linnaeus	KC430096	Pulis et al. (2013)
Haploporidae	*Parasaccocoelium haematocheilum* Besprozvannykh, Atopkin, Ermolenko & Nikitenko, 2014	*Liza haematocheila* (Temminck & Schlegel)	HF548461	Besprozvannykh et al. (2014)
Haploporidae	*Parasaccocoelium mugilid* Zhukov, 1971	*Liza haematocheila* (Temminck & Schlegel)	HF548468	Besprozvannykh et al. (2014)
Haploporidae	*Parasaccocoelium polyovum* Besprozvannykh, Atopkin, Ermolenko & Nikitenko, 2014	*Liza haematocheila* (Temminck & Schlegel)	HF548474	Besprozvannykh et al. (2014)

Litosaccus n. g.

Diagnosis

Body of adult elongate, cylindrical, slightly more than 6× longer than wide. Tegument sparsely spinous. Eyespot pigment diffuse in forebody. Oral sucker terminal, infundibuliform, with small papillae surrounding periphery. Ventral sucker slightly elevated, transversely oval, shorter than oral sucker. Prepharynx distinct. Pharynx subglobular to globular, smaller than oral sucker. Esophagus present. Intestinal bifurcation approximately at second fifth of body length. Caeca two, cylindrical, uneven to subequal, end blindly at approximately last quarter of body. Testis single, subspherical, median, located approximately at level of midbody. External seminal vesicle claviform to saclike. Hermaphroditic sac not well developed, in first quarter of body length, arcuate, elongate-oval, slightly longer than to 1.59 length of pharynx; sac containing internal seminal vesicle, small prostatic bulb, thin-walled

male duct, female duct, and hermaphroditic duct. Genital atrium shallow. Ovary subglobular to globular, medial, pretesticular. Uterus occupies most of hindbody. Vitellarium in two clusters of subglobular to globular follicles, posterolateral to ovary. Eggs numerous, containing developed miracidia with two fused eye-spots. Excretory vesicle I-shaped, bulbous anteriorly, terminating in hindbody. In Mugilidae; in Southwest Pacific Region. *Type- and only species: Paralecithobotrys brisbanensis* Martin, 1974.

Etymology: The Greek *litos* for "simple" and the masculine Greek *saccus* for "sac" refer to the small, relatively simple hermaphroditic sac.

Remarks

The new genus presently accommodates only *Litosaccus brisbanensis* (Martin, 1974) n. comb. that is morphologically most similar to the haploporine genera *Lecithobotrys* and *Pseudolecithobotrys* Blasco-Costa, Gibson, Balbuena, Raga & Kostadinova, 2009 in possessing a vitellarium comprising two grape-like clusters of follicles lateral to the ovary. The new genus can be separated from the two by possessing two uneven caeca, an infundibuliform oral sucker, a small, thin-walled hermaphroditic sac (hermaphroditic sac length/ventral sucker length 57–104% as opposed to over 110%), and shallow genital atrium. Additionally, it can be further differentiated from *Lecithobotrys* in having an elongate, cylindrical body rather than a fusiform to pyriform body and can be further differentiated from *Pseudolecithobotrys* in possessing a subspherical testis rather than an elongate, subcylindrical testis. Martin (1974) originally described *P. brisbanensis* as having a hermaphroditic duct "lined with tiny spines or tubercles," a feature we cannot confirm. Our specimens do not appear to have any spines or tubercles lining the hermaphroditic duct, although he stated that it is best seen in specimens with an everted duct, not present in the specimens we examined.

***Litosaccus brisbanensis* (Martin, 1964) n. comb.**

Syns *Paralecithobotrys brisbanensis* Martin, 1964; *Lecithobotrys brisbanensis* (Martin, 1964) Overstreet & Curran, 2005

Type- and only known host: Mugil cephalus Linnaeus, flathead grey mullet (Teleostei: Mugilidae).

Type-locality: Brisbane River, Queensland, Australia.

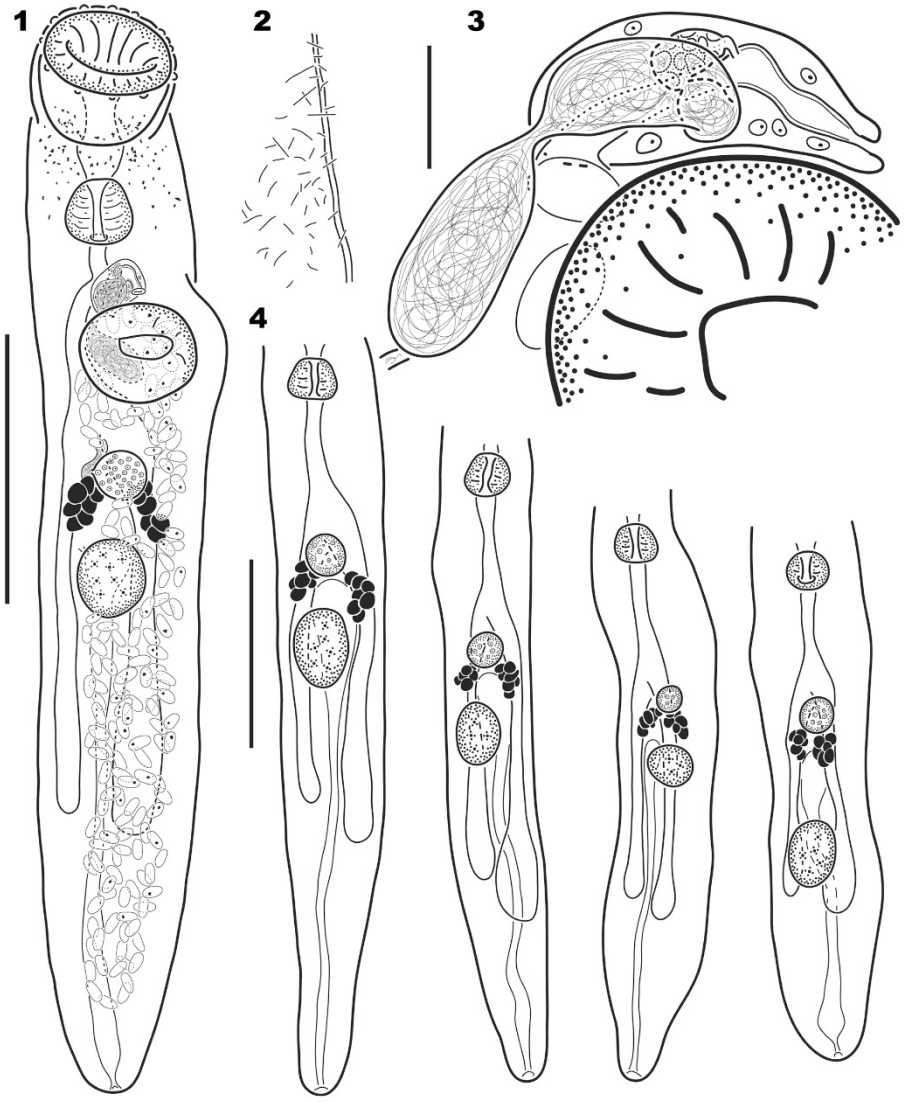
Other localities: Shorncliffe Beach, Bramble Bay, QLD, 27°19′26″S, 153°5′10″E (Fig. 1); Shorncliffe Boat Ramp, Cabbage Tree Creek, QLD, 27°19′47″S, 153°5′11″E (DNA); Brisbane River, Toowong, QLD (27°29′29″S, 152°59′34″E); Wynnum Creek, QLD (27°26′9″S, 153°10′28″E); Redland Bay, QLD.

Site in host: Intestine.

Type-material: Hancock Parasitology Collection, University of Southern California, No. 7112 (presently unable to locate).

Voucher material: Queensland Museum, Brisbane, Australia, G234515–G234522; Harold W. Manter Laboratory Collection, Lincoln, Nebraska, USA P-2014-021.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. KM253765, from 2 identical sequences (2 adult specimens from Cabbage Tree Creek, QLD).



Figures 1–4. *Litosaccus brisbanensis* n. comb. from *Mugil cephalus*. 1, Ventral view; 2, Ventral view of tegumental spines in sinistral margin of forebody; 3, Lateral view of hermaphroditic sac and external seminal vesicle; 4, Ventral view of four other specimens showing variation in the caeca. *Scale-bars:* 1, 4, 500 μm; 2, 3, 50 μm

Description (Figs. 1–4)

[Measurements based on 11 gravid whole-mounts.] Body elongate, cylindrical, 2,048 (1,416–2,256) long, 302 (227–285) wide at second fifth of body length (BL), with width representing 15 (12–19)% of BL. Tegumental spines exceptionally thin, 5–10 (6–13) long. Forebody 563 (339–581) long, representing 27 (23-30)% of BL. Hindbody 1,312 (923–1,575) long, representing 64 (60–70)% of BL. Oral sucker infundibuliform, terminal, 259 (192–267) long, 245

(201–234) wide, with anterior periphery surrounded by ring of approximately 12 small papillae. Ventral sucker 173 (154–192) long, 204 (137–190) wide. Ratio of oral sucker to ventral sucker width 1:0.83 (1:0.67–0.88). Prepharynx 64 (41–88) long. Pharynx subglobular, approximately twice length of prepharynx, 118 (89–128) long, 126 (99–121) wide. Ratio of oral sucker width to pharynx width 1:0.51 (1:0.48–0.60). Esophagus 96 (117–317) long, extending to second fifth of BL, swollen posteriorly. Intestinal bifurcation at or posterior to level of ventral sucker. Caeca long, relatively narrow, uneven to subequal (sinistral caecum longer in all but 1 specimen), more bulbous posteriorly in most specimens, terminating blindly, with posterior-most caecum terminating 481 (293–577) from posterior end, with postcaecal space representing 24 (15–34)% of BL.

Testis single, 151 (113–211) long, 129 (113–163) wide, 270 (210–346) from posterior margin of ventral sucker. Post-testicular space 893 (443–1,074) long, representing 44 (28–48)% of BL. External seminal vesicle claviform to sac-like, 163 (72–158) long, 68 (29–75) wide, dorsal to ventral sucker. Hermaphroditic sac thin-walled, anterodorsal to dorsal of ventral sucker, 112 (109–190) long, 67 (55–89) wide, representing 65 (57–104)% of ventral sucker length and 5 (6–10)% of BL; containing internal seminal vesicle 78 (61–102) long by 38 (24–40) wide, prostatic bulb, female duct, and hermaphroditic duct; male and female ducts unite at anterior third of hermaphroditic sac; hermaphroditic duct muscularized, approximately 1/3 length of hermaphroditic sac. Genital pore medial, 55 (10–56) anterior to anterior margin of ventral sucker.

Ovary globular to subglobular, medial, 91 (67–145) long, 94 (65–109) wide, 101 (17–130) from posterior margin of ventral sucker, 76 (9–227) from anterior margin of testis, posteroventral to ventral to intestinal bifurcation. Uterus emerging from dextral side of ovary, winding anteriorly to or slightly beyond posterior margin of ventral sucker and then winding posteriorly, occupying most of hindbody, with proximal portion filled with sperm. Laurer's canal not observed. Vitellarium in 2 lateral clusters of 7–10 subglobular to spherical follicles 26–30 (24–46) long by 26–29 (23–39) wide, with sinistral cluster 125 (96–162) long, dextral cluster 103 (79–129) long, contiguous or nearly so with posterior margin of ovary, with anterior-most follicle 157 (106–218) from posterior margin of ventral sucker, ventral to caeca. Eggs thin-shelled, numerous, in distal portion of uterus mostly with developed miracidia having eye-spots fused, 40–45 (40–46) long, 24–26 (22–26) wide.

Excretory vesicle I-shaped, bulbous anteriorly, terminating just posterior to ovary, with 1 specimen having well-defined crura extending anteriorly from level of vitelline clusters; pore terminal.

Remarks

Martin's (1974) type-material (originally deposited in the no longer cohesive Hancock Parasitology Collection, University of Southern California) is still missing; we have been unsuccessful in our attempt to find the holotype at the Santa Barbara Museum of Natural History (Pers. comm. Daniel Geiger & Patricia Sadeghian), the Los Angeles County Museum of Natural History (Pers. comm. Joel Martin), and the US National Helminthological Collection (Pers. comm. Patricia Pillit). For consistency we chose to illustrate and measure the same specimen illustrated by Overstreet & Curran (2005) in their chapter in the *Keys to the Trematoda* Vol. 2 (fig. 12.9). The excretory vesicle was described by Martin (1974) as

being Y-shaped, but it is I-shaped in all of our specimens. However, in one of the specimens, the one illustrated (Fig. 1), there are well-defined crura extending from level of the vitelline clusters. These crura are likely collecting branches because each is differentiated from the vesicle by a sphincter. Martin (1974) did not indicate the presence of small papillae surrounding the oral sucker that usually are apparent on many well-fixed trematodes, but the shape of the oral sucker in his illustration and his measurements are consistent with our specimens. Martin (1974) reported the tegument as mostly smooth but with a few spines dorso-anteriorly and immediately posterior to the ventral sucker. Tegumental spines were observed by us in only four of our specimens; two had thin spines sparsely covering the entire tegument and two had only a few spines posterior to the ventral sucker. Presumably, the spines of *L. brisbanensis* are fragile, shallowly embedded, or easily lost and were therefore not observed on most of our specimens because of loss due to fixation, preservation, or handling techniques. Despite these potential differences and based on the size and shape of the body, suckers, reproductive organs, and hermaphroditic sac, we have no doubt that the specimens we collected are conspecific with those of Martin (1974).

Molecular analysis

The DNA sequence fragment amplified encompasses the 3′ end of the 18S gene, the ITS region (ITS1-5.8SITS2), and 1,415 bp of the 5′ end of the 28S gene. No intraspecific variation occurred between the two sequenced specimens of *L. brisbanensis*. The alignment of partial 28S rDNA sequences of *L. brisbanensis* and related species from GenBank was 1,128 characters long with 655 conserved sites, 473 variable sites, and 337 informative sites. The BI analysis of those sequences incorporated the paragonimid *P. westermani* as an outgroup and an ingroup of two species each of atractotrematids and *Cadenatella*, *L. brisbanensis*, and 21 other species of Haploporidae (Fig. 5). The ingroup of the Haploporidae was revealed as a paraphyletic clade. The megasolenine *Hapladena nasonis* Yamaguti, 1970 was well supported as basal to *Cadenatella* spp. and the other haploporids. The position of *Cadenatella* as sister to the non-*Hapladena* haploporids was poorly supported. The 20 other non-*Hapladena* haploporids formed a polytomy consisting of *Forticulcita gibsoni* Blasco-Costa, Montero, Balbuena, Raga & Kostadinova, 2009, *Spiritestis herveyensis* Pulis & Overstreet, 2013, *Capitamitta* spp. + *Parasaccocoelium* spp., and a clade that included two subclades: one comprised of *Intromugil* spp. + *Saccocoelioides* sp. and the other of *Litosaccus brisbanensis* + the Mediterranean haploporines.

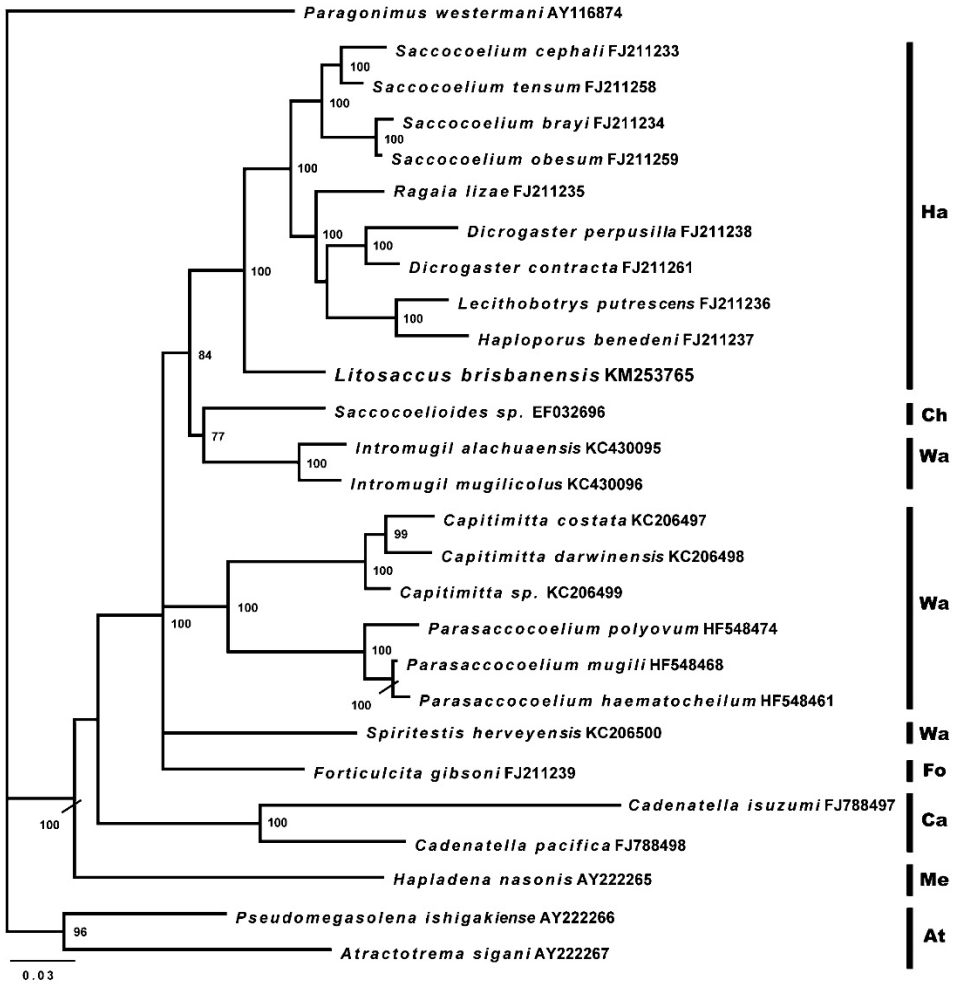


Figure 5. Phylogenetic relationships among members of the Haploporidae resulting from Bayesian inference analysis of partial 28S rDNA sequences (GTR + I + Γ; 1,000,000 generations and a sample frequency of 100) revealing *Litosaccus brisbanensis* n. comb as a haploporine. Support values of < 75% not shown. Vertical bars denote family or subfamily groups. *Abbreviations:* At, Atractotrematidae; Ca, Cadenatellinae; Ch, Chalcinotrematinae; Fo, Forticulcitinae; Ha, Haploporinae; Me, Megasoleninae; Wa, Waretrematinae.

Discussion

Blasco-Costa et al. (2009b) considered *Lecithobotrys brisbanensis* as a *species inquirenda* and stated that it likely did not belong in *Lecithobotrys*; our BI analysis confirms that it does not. We erected *Litosaccus* for *L. brisbanensis*, which has morphological characters in common with the Haploporinae (i.e., vitellarium that is reduced, a uterus that occupies much of the hindbody but does not extend into the forebody, and developed eggs containing miracidia with eye-spots) and is similar to *Lecithobotrys* and *Pseudolecithobotrys*.

In view of the only slight morphological discrepancies between Martin's (1974) specimens and our own, we have little doubt that our specimens are conspecific with those originally described. In the redescription of *I. mugilicolus* by Pulis et al. (2013), they noted that the hermaphroditic duct had a "series of sacs containing a glandular substance" that was observable in living specimens and specimens stored in ethanol, but they were no longer easily discernible after processing for mounting. Similarly, the "tiny spines or tubercles" described by Martin (1973) as lining the hermaphroditic duct of *L. brisbanensis* may not be apparent in our fixed specimens. Thus, additional specimens need to be examined live to confirm the presence or absence of an armed hermaphroditic duct. *Litosaccus* is not an appropriate repository for either of the other two species of *Lecithobotrys* considered *species inquirenda* by Blasco-Costa et al. (2009b), and we agree that both require further data to clarify their generic affinity.

To the best of our knowledge, *L. brisbanensis* may be considered rare, or its host has not been collected when the infection is at its peak intensity. We have examined a total of 46 specimens of *M. cephalus* from the QLD coast (12 in 1984, 18 in 1997, and 16 in 2010) and recovered only a total of 16 specimens, all from the Brisbane/Moreton Bay area. Lester et al. (2009) found that approximately 50% of the individuals of *M. cephalus* they examined had evidence of infection by the blood fluke *Plethorchis acanthus* Martin, 1975 in the Moreton Bay area, while *M. cephalus* from along the New South Wales coast showed no such infection, suggesting the parasite was acquired in Moreton Bay, perhaps in the upper estuary. A similar pattern may occur for infection with *L. brisbanensis* because we recovered the parasite from Moreton Bay drainages only. Additionally, in 2010 we examined 65 individuals of the greenback mullet, *Chelon subviridis* (Valenciennes), flat-tail mullet, *Liza argentea* (Quoy & Gaimard), and silver mullet, *Paramugil georgii* (Ogilby), from Cabbage Tree Creek and the Pine River, which, along with the Brisbane River, empty into Moreton Bay, and we did not find any specimen of *L. brisbanensis*.

In a review of the Haploporidae, Overstreet & Curran (2005) recognized four subfamilies based on morphology: the Chalcinotrematinae (infecting estuarine and freshwater fishes in the New World and Africa), the Haploporinae (with members primarily in mugilids worldwide), the Megasoleninae Manter, 1935 (primarily in marine, reef-associated perciformes), and the Waretrematinae Srivastava, 1937 (in marine, estuarine, and freshwater fishes worldwide but primarily in the Indo-Pacific). Blasco-Costa et al. (2009a) established the Forticulcitinae Blasco-Costa, Balbuena, Kostadinova & Olson, 2009 (with members in mugilids in the Mediterranean Sea and Red Sea) based on a single, compact vitellarium and their BI analysis of partial 28S rDNA sequence data. This is the first phylogenetic hypothesis of the Haploporidae to include a haploporine collected outside of the Mediterranean Sea. *Litosaccus* was resolved as distinct from *Lecithobotrys* but well supported as sister to the Mediterranean haploporines (Fig. 5), confirming that members of the Haploporinae are not restricted to the Mediterranean Sea.

We agree with Pulis & Overstreet's (2013) skepticism of the morphologically defined haploporid subfamilies due to the paucity of molecular data for most genera. Our BI analysis revealed the Waretrematinae to be paraphyletic with *Intomugil* being closer to *Saccocoelioides* Szidat, 1954 and *Spiritestis* Nagaty, 1948 being recovered in the polytomy leading

to the other major haploporid clades, but, at this time, we refrain from making any nomenclatural changes. Besprozvannykh et al. (2014) resurrected *Parasaccocoelium* and demonstrated that the three species they treated formed a well-supported clade with *Capitimitta*, which we recovered as well. However, we are skeptical of their consideration of *Pseudohapladena lizae* Liu & Yang, 2002 as a junior synonym of *Parasaccocoelium mugili* Zhukov, 1971. Liu & Yang (2002) described *Ps. lizae* as having a longer esophagus, smaller eggs, a well-separated ovary and testis, and a more tubular vitellarium.

Bray et al. (2014) used BI analysis of 28S rDNA sequences to demonstrate that *Cadenatella* had previously been misplaced in the Enenteridae Yamaguti, 1958 (Lepocreadioidea Odhner, 1905) and belongs in the Haploporoidea. They noted that with the inclusion of the *Cadenatella* spp. in the Haploporoidea, the Haploporidae was not well resolved because *Hapladena* Linton, 1910 did not cluster with the other members of the family. We also resolved *Hapladena* (the sole representative of the Megasoleninae included in both analyses) outside of the clade containing *Cadenatella* spp. and the rest of the haploporids. The position of *Cadenatella* as the sister group to the rest of the haploporids was not well supported; thus, an important component of future considerations will be whether these taxa belong in the Haploporidae or whether there is a case for recognition of further family level taxa within the Haploporoidea.

The systematics of haploporids still requires considerable resolution. Erecting *Litosaccus* brings the total number of haploporine genera to ten. Four of those genera, Pseudodicrogaster Blasco-Costa, Montero, Gibson, Balbuena & Kostadinova, 2009, Pseudolecithobotrys, Rondotrema Thatcher, 1999, and Unisaccus Martin, 1973, lack a representative DNA sequence. Since all four of those genera also lack a Mediterranean representative, their inclusion in a molecular framework will help clarify the subfamilial relationships within the Haploporidae and help detect the pattern of diversification within the Haploporinae.

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