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Revision of 13 Genera of Haploporidae (Trematoda)

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The University of Southern Mississippi

REVISION OF 13 GENERA OF HAPLOPORIDAE (TREMATODA)

by

Eric Edward Pulis

Abstract of a Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
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ABSTRACT

REVISION OF 13 GENERA OF HAPLOPORIDAE (TREMATODA)

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The Haploporidae is a family of digeneans united by the combination of the possession of a hermaphroditic sac and a single testis or, rarely, two tandem testes. The major divisions in the Haploporidae have been based on the organization, development, and nature of the male and female reproductive systems. Overstreet and Curran (2005) has been the only attempt to organize the genera of the Haploporidae in a subfamilial framework. In the present work the validity of the subfamily Waretrematinae by Overstreet and Curran (2005) is assessed by morphological and molecular methods, based on original descriptions, type and vouchered, specimens and newly collected material for morphology and rDNA sequence data analyses. These analyses conflicted with hypotheses and framework by Overstreet and Curran (2005) in that: (1) Megasoleninae is the basal subfamily within the Haploporidae; (2) Waretrematinae and Haploporinae are not sister groups; (3) species of *Spiritestis* and *Capitimitta*, both previously considered members of *Waretrema*, are not closely related; (4) Waretrematinae was not monophyletic when *Unisaccus* and New Genus 1, new species are included; (5) species of *Unisaccoides* and *Unisaccus* species are closely related; and (6) species of *Intromugil* are allocated to Chalcinotrematinae rather than Waretrematinae. Reports of *Waretrema* were determined to comprise members of three different genera. The morphology of *Intromugil* was accessed by the redescription of *I. mugilicolus* from newly collected material and a new species is described. Species from the Indo-Pacific

region possessing spirally arranged pads in the hermaphroditic duct and a caecum were accessed and required changes to the organization and membership of several genera plus changes to the intergeneric relations within the family based on phylogenetic analysis. Members of the genera *Platydidymus* and *Carassotrema* were assessed and a new species was described. Two new genera of haploporid were diagnosed, a new species was described for each, and phylogenetic relations are estimated. A new genus and new species of Megaperidae are described, and molecular data were provided for three other species. Previously, megaperid species were members of the Apocreadiidae rather than the Haploporidae. Phylogenetic hypotheses based on Bayesian Inference analysis of an alignment of partial 28S gene sequences of haploporids provide a framework for the evaluation of the interrelationships within the Haploporidae.

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A Dissertation
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This dissertation is not intended as a scientific record (see article 8.2, ICZN, International Code of Zoological Nomenclature) for the taxonomic names and nomenclatural acts contained within the dissertation under article 8.3 of the ICZN. This dissertation is not a contribution to the primary scientific literature, nor should it be cited as such.

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CHAPTER I

INTRODUCTION

Members of the Haploporidae Nicoll, 1914 inhabit the gastrointestinal tract of marine, brackish, and freshwater fishes. The defining characters of the family are a hermaphroditic sac and a single testis or, rarely, two tandem testes. Members of the closely related family Atractotrematidae Yamaguti, 1939 also possess a hermaphroditic sac, but they always possess two symmetrical or slightly oblique testes. The most recent treatments of the family included the Megasolenidae Manter, 1935, Waretrematidae Srivastava, 1937, and Hyporhamphitrematidae Machida and Kuramochi, 2000 as synonyms of Haploporidae (Overstreet & Curran, 2005; Blasco-Costa, Balbuena et al., 2009; Pulis et al., 2013; Pulis & Overstreet, 2013). The key by Overstreet and Curran (2005) serves as the basis for the most recent treatment of the genera and subfamilies within the Haploporidae, even though they recognize that there will still be problems in the framework they proposed (Overstreet & Curran, 2005). Subfamilies they considered are Megasoleninae Manter, 1935 (syn. Scorpidicolinae Yamaguti, 1971), Haploporinae Nicoll, 1914 (syns. Dicrogasterinae Yamaguti, 1958 and Unisaccinae Martin, 1973), and Waretrematinae Srivastava, 1937 (syns. Carassotrematinae Skrjabin, 1942, Spiritestinae Yamaguti, 1958, and Phanurinae Liu & Yang, 2002). Overstreet and Curran (2005) also proposed the Chalcinotrematinae Overstreet and Curran, 2005 for those genera possessing an extensive uterus and vitellarium irregularly surrounding the testis or in the hindbody and were confined geographically, largely within the neotropics in freshwater fishes. Based on phylogenetic analysis of some members of the Haploporinae, *Saccocoelioides* sp., and a single species of *Forticulcita* Overstreet, 1982, Blasco-Costa,

Balbuena et al., (2009) proposed the Forticulcitinae because of the non-monophyly of the Haploporinae when *Forticulcita* was included.

Further, molecular sequence data analysis has established that the morphological framework of Overstreet and Curran (2005) has other problems. Pulis and Overstreet (2013) established that *Waretrema* as diagnosed by Overstreet and Curran (2005) was not monophyletic, and members were better allocated to at least three genera. Pulis and Overstreet (2013) found that *Spiritestis* Nagaty, 1948 and *Capitimitta* Pulis and Overstreet, 2013 are probably not the closest relatives to each other, showing that characters used to establish morphological generic affiliations require careful assessment to establish relations. While describing a new species of *Intromugil* Overstreet and Curran, 2005, Pulis et al. (2013) did not provide analysis of the sequence data used to differentiate *Intromugil mugilicolus* (Shireman, 1964) Overstreet and Curran, 2005 from *Intromugil alachuaensis* Pulis, Fayton, Curran, and Overstreet, 2013, presumably because of the poor representation of genera with molecular data available within the sub-familial framework currently accepted, and they hinted at a closer relationship of *Intromugil* to the Chalcinotrematinae than to the Waretrematinae.

Analysis of partial 28S sequence data of members of the family Haploporidae (Pulis & Overstreet, 2013; Blasco-Costa, Balbuena et al., 2009; Blasco-Costa et. al., 2010) has consistently shown that diversity within the family has been underestimated, and characters used for morphological segregation are most likely more plastic than previously considered.

The objectives of this study are to (1) assess the availability of names within selected genera, (2) assess the monophyly of the Waretrematinae, (3) determine the

relations among the subfamilies of the Haploporidae, (4) describe new species, and (5) construct a phylogenetic hypothesis of the relationships among genera.

CHAPTER II

REVIEW OF PISCINE HAPLOPORID (TREMATODA) GENERA WITH ORNATE MUSCULARISATION IN THE REGION OF THE ORAL SUCKER, INCLUDING FOUR NEW SPECIES AND A NEW GENUS

Abstract

Species of the Haploporidae Nicoll, 1914 with elaborate muscularisation of the oral sucker belong in three trematode genera, including three new species and a new genus from the intestine of fishes in Australian waters. *Spiritestis* Nagaty, 1948 is resurrected and *S. herveyensis* n. sp. is described from the mullet *Moolgarda seheli* (Forsskål) collected in Hervey Bay, Queensland, Australia. *Spiritestis herveyensis* differs from *S. arabii* Nagaty, 1948 in that the position of the genital pore is pharyngeal rather than post-pharyngeal and the geographical range is off Australia rather than in the Red Sea. A new genus is proposed for two new species with uniquely ornamented oral suckers, which infect Australian scatophagids. Members of *Capitimitta* n. g. are distinguished from *Waretrema* Srivastava, 1937, species of which have simple oral suckers with six radially arranged anterior muscular lobes, in that their oral sucker is V-shaped with six embedded muscular finger-like structures in the anteroventral portion. The relatively small *C. darwinensis* n. sp., collected from *Selenotoca multifasciata* (Richardson) at Darwin, Northern Territory, Australia, is distinguished from *C. costata* n. sp., collected from *Scatophagus argus* (Linnaeus) in the same locality and *S. multifasciata* off Brisbane, Australia, by having smaller eggs, a vitellarium commencing at a level close to the ventral sucker rather than at greater than one ovarian length posterior to the ventral sucker, and shorter tegumental body spines. Sequence data of an

approximately 2,500 bp region of the 3' end of 18S gene, the entire ITS region and the 5' end of the 28S gene sequence revealed that *Spiritestis* and *Capitimitta* are not as closely related as some morphological features would suggest and probably are not the closest relative of each other. What has been reported as *Waretrema piscicolum* Srivastava, 1937 probably consists of several species, some in different genera, and one, based on material collected by Dr. Masaaki Machida, is proposed as *Spiritestis machidai* n. sp. from *Crenimugil crenilabis* (Forsskål) off Japan. Phylogenetic hypotheses, based on analysis of an alignment of partial 28S sequences with other haploporids, provide a framework for the evaluation of interrelationships within the Haploporidae. These analyses show that (1) *Spiritestis* and *Capitimitta* are supported within the Haploporidae; (2) branches to *Forticulcita* Overstreet, 1982, *Saccocoelioides* Szidat, 1954, *Spiritestis* and *Capitimitta* create a clade that is sister to haploporines from the Mediterranean Sea; (3) the branch to *Saccocoelioides*, S*piritestis* and *Capitimitta* create a polytomy; and (4) the two new species of *Capitimitta,* plus an immature specimen of an unnamed species, form a monophyletic clade. All new taxa were previously published in Pulis and Overstreet (2013).

Introduction

Haploporid trematodes are cosmopolitan parasites of the alimentary tract of fishes characterised primarily by the presence of a hermaphroditic sac and a single testis (Overstreet & Curran, 2005). The organisation of the subfamilies and genera has been called into question by many authors (reviewed by Overstreet & Curran, 2005) and more recently by the proposal of Forticulcitinae Blasco-Costa, Balbuena, Kostadinova, and Olson, 2009. Haploporids have the greatest diversity in the Mugilidae in both the number

of species and genera described, but some also infect members of other fish families. Prior to this study, only two species of haploporids, both placed in *Waretrema* Srivastava, 1937 by Overstreet and Curran (2005), were reported to have an ornamented oral sucker. The type-species for *Waretrema*, the type-genus of Waretrematinae Srivastava, 1937, is *W. piscicolum* Srivastava, 1937. Although this species has been reported six times (Srivastava, 1939; Velasquez, 1961; Gupta & Miglani, 1976; Bilqees, 1980; Machida, 1996; Liu & Yang, 2003), I doubt that any of the subsequent reports represent a species in *Waretrema* as diagnosed by Srivastava. *Waretrema piscicolum* was described in detail by Srivastava (1939) from specimens obtained from *Liza vaigiensis* (Quoy & Gaimard) (reported as *Mugil waigiensis* [Quoy & Gaimard]) in the Arabian Sea, off Karachi, Pakistan. Specimens identified as *W. piscicolum*, or *W. piscicola*, have been reported from *Crenimugil crenilabis* (Forsskål) off Okinawa, Japan (Machida 1996) and a marine fish off the Andaman and Nicobar Islands, India (Gupta & Miglani, 1976). It also has been reported from *Scatophagus argus* (Linnaeus) off Karachi, Pakistan (Bilqees, 1980), off the Philippines (Velasquez, 1961), and in the South China Sea (Liu & Yang, 2003). Nagaty (1948) described *W. arabii* (Nagaty, 1948), as *Spiritestis arabii* Nagaty, 1948, from *Mugil* sp. in the Red Sea. *Spiritestis* Nagaty, 1948 was proposed as a junior synonym of *Waretrema* by Overstreet and Curran (2005) because of the superficial similarity of the oral sucker in *S. arabii* and *W. piscicolum*.

In this study, on the basis of old and new specimens, I concluded that haploporids with ornamentation in the region of the oral sucker are best considered as representing three genera. One is *Waretrema* (*stricto sensu*), one is resurrected, and the last is new. I discuss records of *W. piscicolum* and examine several specimens with an ornate anterior

end and show that *Waretrema*, as defined by Overstreet and Curran (2005), is polyphyletic, resurrect *Spiritestis*, describe two new species in that genus, propose a new genus for three species infecting *Scatophagus argus* and *Selenotoca multifasciata* (Richardson) in Australian waters, and describe two of these species as new. Molecular data obtained from the ITS1, ITS2 and 28S gene fragments are used to support decisions to separate *Spiritestis* from the new genus and describe two species in the new genus. Also, I place these two new species, and an unnamed one, within a phylogenetic framework of a larger group of haploporids using the 28S gene fragment.

Materials and Methods

Specimens of three undescribed species belonging to *Spiritestis* and a new genus, along with other trematodes, were collected from *Moolgarda seheli* (Forsskål), *Selenotoca multifasciata,* and *Scatophagus argus* at several locations in Australian waters during March 2010 using cast-nets. Fish length was measured as total length (TL), extending from the tip of the snout to the end of the tail. Fish names follow those given by FishBase (Froese & Pauly, 2013).

Trematodes were collected following Cribb and Bray (2010) for gastrointestinal species, often skipping the initial examination under a dissecting scope for mullets due to the volume of the intestinal contents. Live worms were rinsed and cleaned in a container with saline, and examined briefly; then most of the saline was removed from the container, and the worms were killed by pouring hot, steaming water (not boiling) over them. This procedure was followed by fixing the worms in 70% ethanol. When the number of specimens of a species allowed, a few additional ones were placed at room temperature directly into 95% ethanol for molecular analysis, and a couple were heat-

killed while under coverslip pressure for the critical examination of ducts. Worms were stained in aqueous alum carmine, Mayer's haematoxylin or Van Cleave's haematoxylin, dehydrated in a graded ethanol series, cleared in clove oil (carmine and Van Cleave's) or methyl salicylate (Mayer's), and mounted permanently in Damar gum. Measurements were taken using a differential interference contrast (DIC) equipped Leica compound microscope using a ProgRes® CapturePro camera (Version 2.8 Jenoptic, Jena, Germany) and software. All measurements are in micrometres unless otherwise noted. Museum abbreviations are as follows: GCRLM, Gulf Coast Research Laboratory Museum; MAGNT, Museum and Art Gallery of the Northern Territory, Darwin, Australia; NSMT, National Science Museum Tokyo; QM, Queensland Museum, Brisbane, Australia; and USNPC, US National Parasite Collection, Beltsville, Maryland.

With regard to the terminology of structures, I want to clarify one matter involving the terminal genitalia reported differently by various authors. The uterus enters the posterior portion of the hermaphroditic sac, also termed "hermaphroditic pouch" by some, (see Overstreet & Curran, 2005) and it continues its path to join the male duct as a hermaphroditic duct or intromittent organ. I do not consider the distal portion of the uterus before entering the hermaphroditic sac a "metraterm" unless there occurs a distinct sphincter, which is usually considerably more muscular than the uterus-proper. This "metraterm" controls or inhibits the passage of eggs into the hermaphroditic sac. I did not encounter such a structure in specimens reported herein. Once the uterus enters the sac, I consider the tube, which is usually quite muscular, as the "female duct", and this duct can, in rare cases (not observed here), be subdivided into two portions by a sphincter forming an internal metraterm.

For comparisons with previously labelled and deposited specimens, I examined the following from the USNPC: *Spiritestis arabii* (USNPC 038164.00, voucher [Nagaty, 1948]), *Spiritestis arabii* (USNPC 059541.00, paratype [Nagaty, 1948]) and *Waretrema piscicola* (USNPC 039476.00, labelled as topotype [Velasquez, 1961]); and from the NSMT: *W. piscicola* (NSMT Pl- 3841, 4700 1/2, 4700 2/2, 4705, and 4731 [Machida, 1996]). No type of *W. piscicolum* Srivastava, 1937 was designated at the time of publication.

Genomic DNA was isolated using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the instructions provided. DNA fragments *c*.2,500 base pairs (bp) long, comprising the 3' end of the 18S nuclear rDNA gene, internal transcribed spacer region (including ITS1 + 5.8S + ITS2), and the 5' end of the 28S 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 primers ITSF (5' - CGCCCGTCGCTACTACCGATTG-3') or LSU5 (5'-TAGGTCGACCCGCTGAAYTTAAGCA-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’-CAACTTTCCCTCACGGTACTTG-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 were assembled using Sequencher™ (GeneCodes Corp., Ann Arbor, Michigan, USA, Version 4.10.1) and submitted to GenBank. Previously published 28S ribosomal RNA gene sequences of *Atractotrema sigani* Durio and Manter, 1969, *Dicrogaster contracta* Looss, 1902, *D. perpusilla* Looss, 1902, *Forticulcita gibsoni* Blasco-Costa, Montero, Balbuena, Raga, and Kostadinova, 2009, *Hapladena nasonis* Yamaguti, 1970, *Haploporus benedeni* Looss, 1902, *Lecithobotrys putrescens* Looss, 1902, *Pseudomegasolena ishigakiense* Machida & Kamiya, 1976, *Saccocoelioides* sp. of Overstreet and Curran (2005), *Saccocoelium brayi* Blasco-Costa, Montero, Balbuena, Raga, Kostadinova, and Olson 2009, *S. cephali* Blasco-Costa, Montero, Gibson, Balbuena, Raga, and Kostadinova, 2009, *S. obesum* Looss, 1902, *S. tensum* Looss, 1902, and *Paragonimus westermani* (Kerbert, 1878) Braun, 1899 were used for comparison (see Table 1 for all accession numbers and host information). Sequences were aligned using the ClustalW application in the BioEdit program, Version 7.0.9 (Hall, 1999). The alignment was further refined by eye and trimmed to the shortest sequence on both 5' and 3' ends. The resulting alignment utilised 14 haploporids and the two atractotrematids *Pseudomegasolena ishigakiense* and *Atractotrema sigani*, with *P. westermani* as the outgroup, and it was 1,204 characters long, including gaps, with 790 sites conserved, 411 sites variable, and 276 sites informative. Phylogenetic analysis of the data was performed using Bayesian inference (BI) with MrBayes 3.1.2 software (Huelsenbeck & Ronquist,

Table II.1

List of species, hosts, origins, and GenBank accession numbers of sequences used in this study

Species	Host species	Country	Locality	28S
Paragonimus westermani	Experimental host	India	Meghlaya	DQ836244
Atractotrema sigani	*Siganus lineatus*	Australia	Lizard Island	AY222267
Hapladena nasonis	*Naso unicornis*	Australia	Lizard Island	AY222265
Pseudomegasolena ishigakiense	*Scarus rivulatus*	Australia	Heron Island	AY222266
Dicrogaster contracta	*Liza aurata*	Spain	Santa Pola	FJ211261
Dicrogaster perpusilla	*Liza ramado*	Spain	Santa Pola	FJ211238
Forticulcita gibsoni	*Mugil cephalus*	Spain	Santa Pola	FJ211239
Haploporus benedeni	*Liza ramado*	Spain	Santa Pola	FJ211237
Lecithobotrys putrescens	*Liza saliens*	Spain	Ebro Delta	FJ211236
Saccocoelium brayi	*Liza saliens*	Spain	Ebro Delta	FJ211234
Saccocoelium cephali	*Mugil cephalus*	Spain	Ebro Delta	FJ211233
Saccocoelium obesum	*Liza ramado*	Spain	Ebro Delta	FJ211259
Saccocoelium tensum	*Liza aurata*	Spain	Santa Pola	FJ211258
Saccocoelioides sp.	Poecilidae	Nicaragua		EF032696
Capitimitta arwinensis n. sp.	*Selenotoca multifasciata*	Australia	Northern Territory, Darwin	KC206498
Capitimitta costata n. sp.	*Selenotoca. multifasciata*, *Scatophagus argus*	Australia	Brisbane, Queensland (*S. multifasciata*); Darwin, Northern Territory (*S. argus*)	KC206497
Capitimitta sp.	*Selenotoca multifasciata*	Australia	Causeway Lake, Queensland	KC206499
Spiritestis herveyensis n. sp.	*Moolgarda seheli*	Australia	Hervey Bay, Queensland	KC206500

2001; Ronquist & Huelsenbeck, 2003). The best nucleotide substitution model was estimated with jModeltest Version 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) 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 1,780 estimated by plotting the log-probabilities against generation and visualising plateau in parameter values (*sump burnin=1780*), and nodal support was estimated by

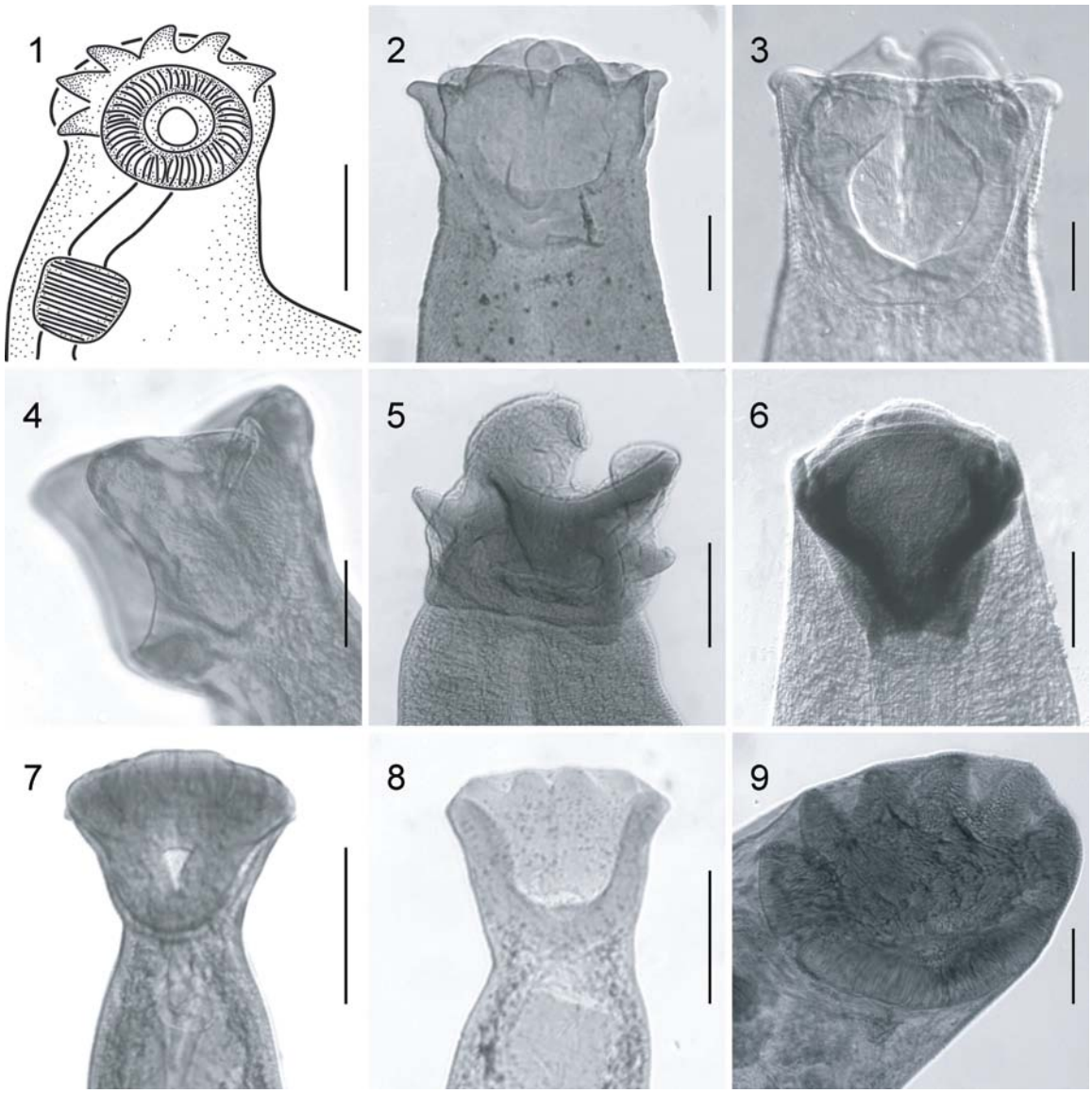
posterior probabilities (*sumt*) (Huelsenbeck et al., 2001), with all other settings left as default.

Waretrema Srivastava, 1937

Diagnosis: (Figure 1)

Body fusiform. Eye-spot pigment unknown but assumed present. Tegument spinous, with spines on forebody. Oral sucker subspherical with 6 separate anterodorsal conical lobes (arranged radially in relation to anterior half), ventral, with spines like those of tegument. Ventral sucker larger than oral sucker, in anterior third of body. Prepharynx present. Pharynx well developed. Oesophagus relatively long. Intestinal bifurcation posterior to ventral sucker. Caeca elongate, saccular, ending blindly anterior to ovary. Testis singular, ovoid, in posterior third of body. External seminal vesicle present. Hermaphroditic sac contains internal seminal vesicle, pars prostatica with surrounding prostatic gland cells, female duct and hermaphroditic duct; female duct and hermaphroditic duct about equal in length. Ovary slightly dextral, contiguous with testis to pretesticular. Laurer's canal present. Canicular seminal receptacle present. Vitellarium composed of 10 elongate spindle-shaped follicles, extends anteriorly to mid-body and posteriorly close to posterior extremity of body. Uterus pre-ovarian; eggs few, medium-sized, thin-shelled. Excretory vesicle Y-shaped, bifurcates at posterior level of testis; pore subterminal. Parasites of Mugilidae. Type and only recognised species: *Waretrema piscicolum* Srivastava, 1937.

Etymology: The genus was named by Har Dayal Srivastava for Mr. F. Ware, Director, Imperial Veterinary Institute, Mukteshwar-Kumaon, Uttarakhand, India. Because of the Greek neuter "trema" for hole, the genus is treated as neuter in gender.



Figures II.1-9. Oral suckers of *Waretrema*, *Spiritestis*, and *Capitimitta*. 1. *Waretrema piscicolum*, illustration of the anterior end, redrawn from Srivastava (1939); 2. *Spiritestis arabii*, ventral view of specimen heat-killed while under pressure; 3. *Spiritestis herveyensis* n. sp., ventral view of specimen heat-killed without pressure; 4. *S. herveyensis*, lateral view of specimen heat-killed without pressure; 5. *S. machidai* n. sp., extended oral sucker of specimen killed while under pressure; 6. *S. machidai*, contracted oral sucker of specimen killed while under pressure; 7. *Capitimitta darwinensis* n. sp., oral sucker of specimen heat-killed without pressure; 8. *C. darwinensis*, oral sucker of specimen heat-killed while under pressure; 9. *Capitimitta* sp., oral sucker of worm labelled as "topotype" of *Waretrema piscicolum* collected from *Scatophagus argus* by Velasquez (1961), specimen killed while under pressure. *Scale-bars* 1, 250 µm; 2-9, 100 µm.

Waretrema piscicolum Srivastava, 1937

Syns Waretrema piscicola Srivastava, 1937; *W. piscicola* of Srivastava (1939)

Type- and only known host: *Ellochelon vaigiensis* (Quoy & Gaimard, 1825), squaretail mullet (Mugilidae).

Etymology: The Latin adjectival name *piscicolum* refers to "dwelling in" a fish and corresponds with the neuter generic name.

Description: (Figure 1) With characters of genus.

Remarks

Waretrema piscicolum was first named *W. piscicola* in an abstract presented by Srivastava (1937) that included enough information to separate both species and genus from related haploporid taxa at the time. Soon after, Srivastava (1939) provided the description in more detail. In the abstract, Srivastava (1937) recorded the host as *Trichiurus mutieus* Gray, which I assume to be a misspelling of *Trichiurus muticus* Gray, currently considered to be *Eupleurogrammus glossodon* (Gray). With the full description, Srivastava (1939) corrected the identification of the fish from the Arabian Sea to *Liza vaigiensis* (Quoy & Gaimard) (reported as *Mugil waigiensis* Quoy & Gaimard). Srivastava's (1939) description and illustration are clear and coherent, but subsequent reports (Velasquez, 1961; Gupta & Miglani, 1976; Bilqees, 1980; Machida, 1996; Liu & Yang, 2003) of *W. piscicolum* are suspect and will be discussed under other sections. The two most definitive features of *Waretrema* that separate it from other genera of haploporids are (1) the possession of six radially arranged lobes located anterior to the oral sucker that are covered with spines resembling those of the body tegument and (2) the few elongate vitelline follicles.

The most recent diagnosis of the *Waretrema* by Overstreet and Curran (2005) included members of both *Spiritestis* and a new genus reported herein, which necessitate the narrowing of their concept to that diagnosed above. The oral area of *W. piscicolum* is very unusual for a haploporid, and this unusual appearance is what I believe led to a history of attributing specimens and species to this genus based on superficial characters. Additionally, when specimens are fixed under coverslip pressure, the oral sucker area becomes compressed, making the anterior end of different species appear similar. The illustration of, and description by, Srivastava (1939) is clear, concise and thorough, and there is no evidence to support the possibility that the oral sucker does not have six projections anterior to it. I have provided photomicrographs (Figures 2-9) of species of *Spiritestis* and the new genus and have redrawn the oral area of *W. piscicolum* (Figure 1). Based on these comparisons, I believe that variations in the oral sucker in specimens caused authors to treat all as *W. piscicolum*. For example, when I initially examined live specimens of *Spiritestis*, I thought that I was dealing with *Waretrema*, although recognising others as a new genus of atypical haploporids. The differentiation of *Waretrema* from other genera will be discussed below under *Spiritestis* and the new genus.

Spiritestis Nagaty, 1948

Amended diagnosis: Body elongate. Anteriormost portion of worm deeply cleft, with origin of cleft near level of prepharynx origin. Eye-spot pigment dispersed from levels of oral sucker to ventral sucker, densest between pharynx and oral sucker. Tegument spinous. Oral sucker terminal, with 6 muscular lobes arranged in 3 distinct pairs; first pair forms ventral or anterior rim of oral sucker with slight cleft medially;

second pair dorsal to first pair and extends laterally; third pair dorsal and extends anteriorly. Mouth subterminal, opens ventrally. Prepharynx relatively long. Pharynx pyriform. Oesophagus longer than pharynx. Ventral sucker slightly elevated, without any specialisation, located quarter to third of body length from anterior end. Intestinal bifurcation near posterior margin of ventral sucker. Caeca sac-like to relatively long, narrow, end blindly. Testis longer than wide, in hindbody close to posterior end of caeca, with post-testicular field not more than 10% of body length (BL). External seminal vesicle elongate, sinuous, longer than internal seminal vesicle. Hermaphroditic sac elongate, arcuate. Ovary pretesticular. Vitellarium with numerous (>50) small follicles, located between level slightly anterior to ovary and post-testicular region. Uterus pre-ovarian, posterior to genital pore. Eggs thin-shelled, non-operculate; miracidium lacks pigmented eye-spots. Lymphatic system present in forebody. Excretory vesicle weakly Y-shaped, extends to ovarian region; pore terminal. In Mugilidae; in Indo-Pacific. Type-species *Spiritestis arabii* Nagaty, 1948.

Etymology: Nagaty (1948) described *Spiritestis* based on *S. arabii* as having a single, elongate, more-or-less superficially spiralled testis, but he did not provide an etymological origin of the name. I consider the name a combination of the Latin feminine *spira*, meaning coil or twist, and the Latin *testis* and consider the name masculine, since "testis" is clearly masculine.

Remarks

The combination of morphological features including a spinose tegument, the possession of a hermaphroditic sac, a single testis and a Y-shaped excretory vesicle makes members of this genus fully consistent with the diagnosis of the Haploporidae (see

Overstreet & Curran, 2005), even though the genus was originally placed in the Lepocreadiidae Odhner, 1905 by Nagaty (1948). I resurrect the available name *Spiritestis* because it differs significantly from *Waretrema*. Members of *Waretrema* have the oral sucker composed of six conical, muscular, independent, retractable lobes directed anteriorly (Figure 1). However, in specimens of *Spiritestis*, the oral sucker bears three pairs of lobes (Figures 2-6, 12, 14) of variable mobility in live material, which, in contracted, fixed specimens, remains distinct and are not retracted into the oral sucker even when the latter is withdrawn into the body. Nagaty (1948) stated that there were four lobes in *S. arabii*; presumably, he was referring to the second and third pairs. Based on my examination of two of his specimens, I found that the first pair was inconspicuous because of the contracted nature of the specimens. In these, there was a noticeable thinning of what I consider to be the first pair, as in my Red Sea specimens. Other features that distinguish specimens of *Spiritestis* from *Waretrema* include (1) a pyriform rather than ovoid pharynx, (2) long tubular rather than saccate caeca, (3) numerous small vitelline follicles rather than a few long, relatively large, tubular follicles, and (4) a delicate Y-shaped excretory vesicle extending to the ovary rather than bifurcating at ovarian level and extending at least to the level of the ventral sucker. *Spiritestis* specimens have several features which are odd for a waretrematine, i.e., a long, sinuous external seminal vesicle, a very delicate Y-shaped excretory vesicle and numerous small vitelline follicles. The testis is not spiralled as the name suggests, but it is usually elongate and may give the appearance of being twisted in specimens under pressure or not heat-killed. Other members of the family Haploporidae that exhibit these characters

are in the subfamily Megasoleninae Manter, 1935, suggesting *Spiritestis* may occupy a basal position within the Waretrematinae.

Spiritestis arabii Nagaty, 1948

Syn Waretrema arabii (Nagaty, 1948) Overstreet & Curran, 2005

Type-host: Unidentified *Mugil* sp. known locally as "Boory or Arabi" (Mugilidae).

Other hosts: *Crenimugil crenilabis* (Forsskål), fringlip mullet (Mugilidae); *Moolgarda seheli* (Forsskål), bluespot mullet (Mugilidae).

Type-locality: Red Sea.

Other locality: Off Eilat, Israel, Red Sea.

Material examined: *Spiritestis arabii* Nagaty, 1948 (USNPC 059541.00, paratype; USNPC 038164.00, voucher); 5 voucher specimens from Red Sea collection, 2 from *Crenimugil crenilabis* and 3 from *Moolgarda seheli*, collected from the Gulf of Aqaba, Red Sea, Eilat, Israel, by Ilan Paperna USNPC 106213.00-106215.00.

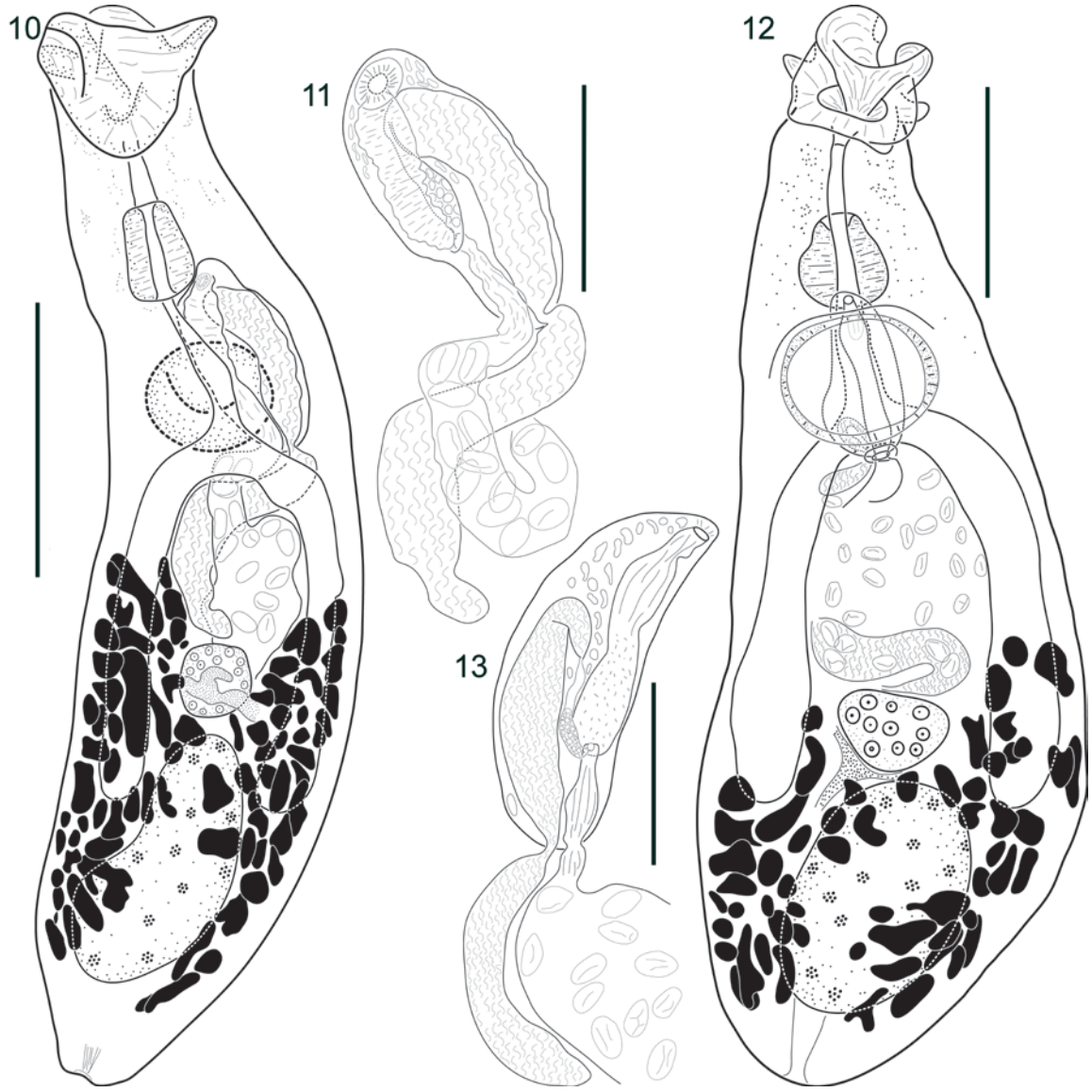
Description: (Figures 2,10-11, Table 2)

Based on 5 gravid specimens collected by Ilan Paperna killed under varying degrees of coverslip pressure with heat. Body elongate, ellipsoidal, 2,371-3,249 long, 346-606 wide, with width 14-26% of BL. Forebody 710-926 or 26-31% of BL. Hindbody 1,389-2,155 or 58-66% of BL. Tegument spinous, with spines 8-10 long in forebody. Eye-spot pigment dispersed. Oral sucker (Figure 2) subterminal, with mouth opening ventrally, 229-368 long, 229-397 wide, with 6 muscular lobes; first pair of lobes ventral and anterior to mouth, with weakly M-shaped extension of anterior oral sucker rim projecting ventrally; second pair dorsal to first pair, extending laterally, forming widest

part of oral sucker apparatus; third pair dorsal to second and extending anteriorly as anteriormost extension of entire worm, flattened dorsoventrally, with total width about same as first pair, overlapping slightly along median junction, uniting posterior to level of anterior margin of first pair. Ventral sucker slightly elevated, 212-282 long, 210-296 wide. Prepharynx 107-297 long. Pharynx pyriform, 137-209 long, 147-181 wide, widest in posterior half, with length 88-139% of width and 55-195% of prepharynx length. Oesophagus 322-482 long, extending to near level of posterior margin of ventral sucker. Intestinal bifurcation 851-1165 from anterior end or 33-41% of BL. Caeca long, tubular, terminating blindly 572-887 from posterior end; postcaecal field 22-28% of BL.

Testis elongate, medial, slightly pointed at posterior end, 713-803 long, 215-251 wide, 608-1,211 from posterior margin of ventral sucker, 42-266 from posterior end of body or post-testicular field 2-9% of BL. External seminal vesicle 444-888 long, 85-137 wide, sinuous, extending posteriorly to near ovary, often obscured by eggs. Hermaphroditic sac thick-walled, arcuate to straight, passing dorsal to ventral sucker, 415-557 long, 163-222 wide, containing internal seminal vesicle measuring 297-434 long, 82-106 wide in posterior region, male duct arising from anterior region of internal seminal vesicle, and pars prostatica which unites with female duct at roughly middle of sac and forms hermaphroditic duct; hermaphroditic duct strongly muscularised, S-shaped, about half length of hermaphroditic sac. Genital pore medial, 100-151 anterior to ventral sucker, 571-785 from anterior extremity or 22-26% of BL.

Ovary medial, 158-205 long, 110-162 wide, 413-609 posterior to ventral sucker, 16-412 anterior to testis. Uterus confined between levels of ovary and slightly posterior to ventral sucker, proximal portion filled with sperm. Laurer's canal not observed.



Figures II. 10-13. Spiritestis arabii and *Spiritestis machidai* 10. *S. arabii*, dorsal wholemount, not all eggs or vitelline follicles illustrated, specimens killed while under pressure; 11. *S. arabii*, hermaphroditic sac of same specimen in Figure 10; 12. *S. machidai* n. sp. ventral wholemount, not all eggs or vitelline follicles illustrated, specimen killed while pressure; 13. *S. machidai* n. sp. hermaphroditic sac, specimen killed while pressure. *Scale-bars* 10,12, 600 µm; 11,13, 300 µm.

Vitellarium follicular; follicles numerous, more than 100, ovoid, distinct, appear as extensive dendritic masses when under pressure, commencing near level of ovary, 243-578 dorsal to ventral sucker, densest when surrounding caeca, absent in area between

testis and ovary, confined to near tegumental surface, terminating 57-110 from posterior end. Eggs thin-shelled, 61-70 long, 33-42 wide, with those in distal uterus not containing miracidium with pigmented eye-spots.

Lymphatic system not observed. Excretory vesicle slightly Y-shaped, bifurcating dorsal to ovary, arms extending slightly anterior to anterior margin of ovary; excretory pore terminal.

Remarks

Examination of two specimens of *S. arabii* (USNPC 038164.00 voucher and USNPC 059541.00 paratype) collected by Nagaty revealed that, even when the oral sucker apparatus was contracted, its state was similar to those I described from my material collected from *Crenimugil crenilabis* and *Moolgarda seheli* in the Red Sea. These specimens were fixed a few decades ago, with extreme coverslip pressure being applied to the specimens from *M. seheli* and moderate pressure to those from *C. crenilabis*, making them unsuitable for comparison with unflattened specimens from recent collections. This species will be discussed under the new species of *Spiritestis*. I encourage the recollection of this species and a redescription.

Spiritestis herveyensis n. sp.

Type- and only host: *Moolgarda seheli* (Forsskål), bluespot mullet (Mugilidae).

Type-locality: Mouth of Beelbi Creek, Hervey Bay, Queensland, Australia (25°14'48"S, 152°40'02"E).

Other locality: Eli Creek, Queensland, Australia (25°15'45"S, 152°48' 28"E).

Site of infection: Intestine.

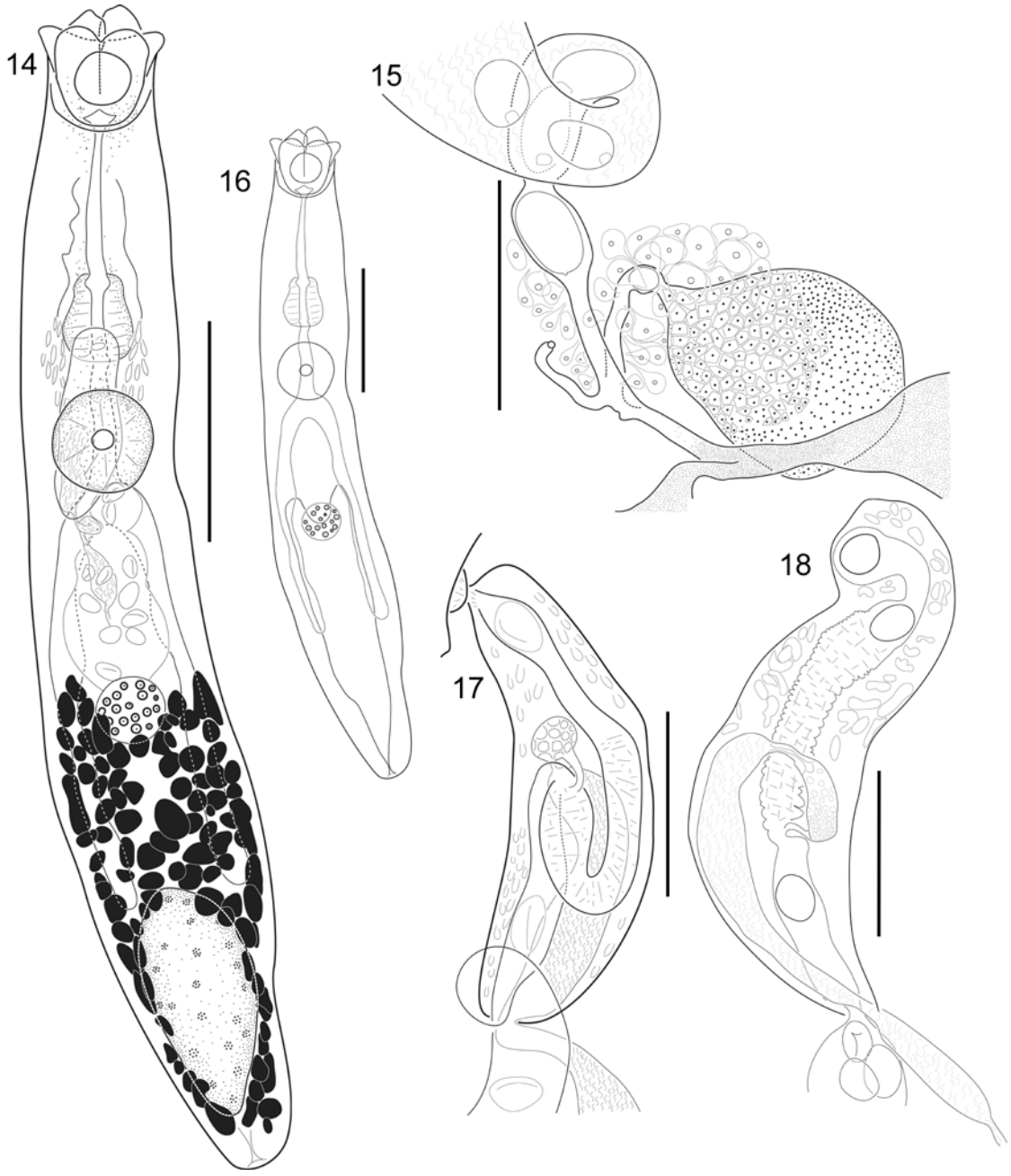
Type-material: Holotype QM G234006; paratypes QM G234007, G234008, USNPC 106216.00-106218.00 (including one flattened specimen USNPC 106217.00 and one lateral mount USNPC 106218.00); *representative DNA sequences* partial 18S, entire ITS region, partial (D1-D3) 28S: GenBank accession no. KC206500, from two identical sequences from Beelbi Creek, QLD.

Etymology: The Latinised, adjectival, masculine name refers to Hervey Bay, from which the type material was collected.

Description (Figures 3-4, 14-18; Tables 1-4)

Measurements based on 6 gravid, unflattened, wholemount specimens; measurements of holotype below and of entire series in Table 2. Body elongate, ellipsoidal, 2,629 long, 447 wide, with width 17% of BL, widest in posterior half of body. Tegument spinose; spines 7-9 long (on forebody of flattened specimen), becoming progressively shorter and more sparse posteriorly. Eye-spot pigment dispersed in anterior third of body. Oral sucker (Figures 3-4, 14) terminal, with 6 muscular lobes (3 pairs), 252 long, 279 wide at widest lateral point of outermost lobe pair (second pair); first pair of lobes less distinctive than other pairs, in form of rounded 'm' anterior to mouth, forms dorsoventral rim of oral sucker; second pair extends laterally and slightly anterior to first pair, forming widest part of oral sucker apparatus; third pair dorsal, about as narrow as first but with sharper 'm' , with deep cleft extending to near mouth; second and third pairs of lobes move independently of other pairs in life. Mouth subterminal, opens ventrally. Ventral sucker slightly elevated, circular in outline, 236 long, 231 wide, with anterior margin 824 from anterior most extremity or 31% of BL. Hindbody 1,563 or 59% of BL. Prepharynx 349 long, with small atrium proximally. Pharynx pyriform, 198 long, 159

wide, widest in posterior half. Oesophagus 365 long, extending posteriorly to near level of posterior



Figures II.14-18. Spiritestis herveyensis n. sp. 14. Ventral wholemount, not all eggs or vitelline follicles illustrated; 15. ovarian complex of specimen killed while under pressure; 16. ventral wholemount showing extent of excretory vesicle; 17. hermaphroditic sac of lateral mount; 18. hermaphroditic sac of specimen killed with coverslip pressure. *Scale-bars* 14,16, 400 µm; 15, 100 µm; 17,18, 200 µm.

Testis elongate, medial, slightly pointed at posterior end, 524 long, 254 wide, 913 from posterior margin of ventral sucker, with post-testicular field 127 or 5% of BL. External seminal vesicle 217 long, 62 wide, sinuous, often obscured by eggs. Hermaphroditic sac (Figures 14, 17-18) thick-walled, arcuate to straight, usually somewhat dextral, passing dorsal to ventral sucker, 425 long, 153 wide, containing internal seminal vesicle in dextroposterior region measuring 209 long, 42 wide, male duct arising from anterior region of internal seminal vesicle, pars prostatica looping ventral to hermaphroditic duct and uniting with female duct to form hermaphroditic duct roughly in middle of sac; hermaphroditic duct strongly muscularised, U- to S-shaped, with total length about length of hermaphroditic sac. Genital pore medial, anterior to ventral sucker, in pharyngeal region, 732 from anterior extremity or 28% of BL.

Ovary (Figure 11) medial, circular to triangular in outline, 136 long, 159 wide, 416 from posterior margin of ventral sucker, 359 from testis. Laurer's canal opens dorsally between levels of ovary and testis. Mehlis' gland slightly anterior to ovary. Vitellarium follicular; follicles numerous, more than 100, commencing near level of ovary about 400 posterior to ventral sucker, densest where surrounding caeca, absent in area between testis and ovary, confined to near tegumental surface, terminating 63 from posterior end; vitelline reservoir ventral to ovary. Uterus confined between levels of ovary and ventral sucker, with proximal portion filled with sperm. Eggs thin-shelled, 61-65 long, 32-34 wide, with those in distal uterus not containing miracidium with pigmented eye-spots.

Lymphatic system consists of 2 large tubes; canals lateral and parallel to prepharynx, terminate near level of ventral sucker, associated with numerous gland-cells

near pharynx. Excretory vesicle slightly Y-shaped, bifurcates dorsal to ovary, terminates slightly anterior to ovary; excretory pore terminal.

Remarks

Based on the combination of features, such as a spinose tegument, hermaphroditic sac and single testis, the new species is consistent with the Haploporidae. The presence of small and numerous vitelline follicles, a pyriform pharynx, tubular caeca and a sinuous external seminal vesicle, in addition to the nature of the oral sucker, places the new species in *Spiritestis*.

Spiritestis herveyensis n. sp. can be differentiated from *S. arabii* by its geographical location, with *S. herveyensis* being from Australian waters and *S. arabii* from the Red Sea. In terms of morphology, the position of the genital pore in *S. herveyensis* is anterior to the posterior margin of the pharynx rather than being at the posterior margin of the pharynx or postpharyngeal; and the third pair of oral lobes (the most anterior and dorsal) in *S. herveyensis* (Figure 3) is almost conical rather than being dorsoventrally flattened and leaf-like (Figure 2).

Spiritestis machidai n. sp.

Syn Waretrema piscicolum of Machida (1996); also redrawn in Figure 12.23 from same collection by Overstreet and Curran (2005).

Type- and only known host: *Crenimugil crenilabis* (Forsskål), fringlip mullet (Mugilidae).

Type-locality: Off Nago, Okinawa Prefecture, Japan.

Material examined (originally identified as *Waretrema piscicolum*): Holotype herein designated from four specimens as the one circled with a diamond pen on slide NSMT Pl-4731; paratypes NSMT Pl- 3841, 4700 1/2, 4700 2/2, 4705, 4705 2/5, 4731. *Description*: (Figures 5-6, 12-13).

Refer to that by Machida (1996; Figure 1).

Remarks

I examined 21 specimens collected by Machida, including the 10 specimens used for the description of *Waretrema piscicolum* by Machida (1996). The specimens examined possess features such as the three pairs of oral lobes, pyriform pharynx and numerous small vitelline follicles that are consistent with species of *Spiritestis*. I consider these specimens to represent a distinct species. *S. machidai* n. sp. (Figures 5-6) can be differentiated from both *S. arabii* and *S. herveyensis* n. sp. (Figures 2-4) by its larger and more elaborate oral sucker; furthermore, in *S. machidai*, the first pair of muscular lobes is directed posteriorly from the oral opening forming a 'W' at the ventroposterior margin of the oral sucker, whereas in *S. arabii* and *S. herveyensis* the first pair of muscular lobes are directed anteriorly and M-shaped. Even though the specimens of *S. machidai* examined were fixed with considerable coverslip pressure, they appear to show that the female duct unites with the male duct to form the hermaphroditic duct more posteriorly in the hermaphroditic sac than in *S. arabii* or *S. herveyensis*. Machida (1996) stated that the tegument of the Japanese specimens was smooth; upon review of the specimens, I determined that many had a few spines in the area around the oral and ventral suckers and assumed that some spines had either been shed or dissolved during fixation or slide preparation. Also, his Figure 1 (Machida, 1996) shows the vitellarium to be larger with

fewer follicles than I observed; this difference probably resulted from the considerable pressure applied to these specimens, distorting some features. *S. machidai* is in need of an amended description based on fresh material that has been killed with hot water without pressure and on accompanying material providing molecular data, preferably including the ITS region and 3' end of the 28s gene.

Capitimitta n. g.

Diagnosis: Body fusiform, elongate, with distinct constriction immediately posterior to oral sucker, widest posterior to ventral sucker, tapers posteriorly. Eye-spot pigment dispersed in forebody, densest between pharynx and oral sucker. Tegument spinose, but spines sparse in area between oral and ventral suckers on ventral surface. Oral sucker specialised, V-shaped, ventroterminal, lies in transverse diagonal plane to body, with anterodorsal margin flat and mouth near posterior margin; anterior region of oral sucker possesses 6 muscular structures in 3 symmetrical pairs; outer pair forms left and right margins of sucker, fanning out to much greater size when extended (like fingers in a mitten), with ventral surface of oral sucker muscular without spines. Ventral sucker smaller than oral sucker. Prepharynx distinct but appearance dependent on orientation of oral sucker on ‘neck’-region. Oesophagus ranges from indistinct to longer than pharynx. Caeca moderately long, sac-like, terminate near mid-body. Testis slightly longer than wide, located near mid-hindbody. External seminal vesicle sac-like, subspherical. Hermaphroditic sac long, J-shaped, dorsal to ventral sucker, terminates slightly posterior to ventral sucker. Genital pore median, anterior to ventral sucker. Ovary contiguous with anterior margin of testis. Vitellarium follicular; follicles few (< 15), relatively large, elongate, tube-like. Uterus anterior to ovary, posterior to hermaphroditic sac. Eggs

relatively few, with miracidium lacking pigmented eye-spots. Excretory vesicle Y-shaped; pore terminal. In Scatophagidae; in Indo-West Pacific Region. Type-species *Capitimitta darwinensis* n. sp.

Etymology: The name *Capitimitta* is constructed from the Latin "capitalis," referring to the anterior end of the worm and the Medieval Latin feminine "mitta," referring to the mitten covering the muscular structures in the oral sucker.

Remarks

Capitimitta n. g. fits within the Haploporidae based on the morphological features listed by Overstreet and Curran (2005), with an emphasis on the possession of a hermaphroditic sac, a single testis, a spinose tegument and a Y-shaped excretory vesicle. The specialised nature of the oral sucker separates it from other haploporid genera, except for *Spiritestis* and *Waretrema*. *Capitimitta* is most similar to *Waretrema,* but can be distinguished from it by the nature of the oral sucker. In specimens of *Waretrema,* the oral sucker (Figure 1) consists of a subspherical oral sucker with six anterodorsal, radially arranged, conical and retractable lobes with a spinose tegument, and the host is a mugilid (Srivastava, 1939). In members of *Capitimitta*, the oral sucker (Figures 7-9, 19, 20, 24, 27, 29) is basically V-shaped and perched on the 'neck' facing ventrally, with the surface of the sucker being smooth. When fixed without pressure, the leading edge of the oral sucker has six humps which represent the anterior region of the muscular structures. The oral sucker in both extended and contracted live worms resembles the fingers in a mitten, giving a webbed appearance. Even in flattened specimens, there is no indication of a subspherical oral sucker (Figures 8-9, 24); in such flattened specimens, the oral sucker has the appearance of a large cup with the bottom rim and muscular structures embedded

within the anterior region. *Capitimitta* superficially resembles *Spiritestis* in that its species possess a complex oral sucker. The oral sucker of *Spiritestis* spp. also has six structures, but they are independent of one another. When fixed under pressure, differences in the oral suckers of members of all three genera are not fully apparent because superficially they appear similar.

Capitimitta darwinensis n. sp.

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

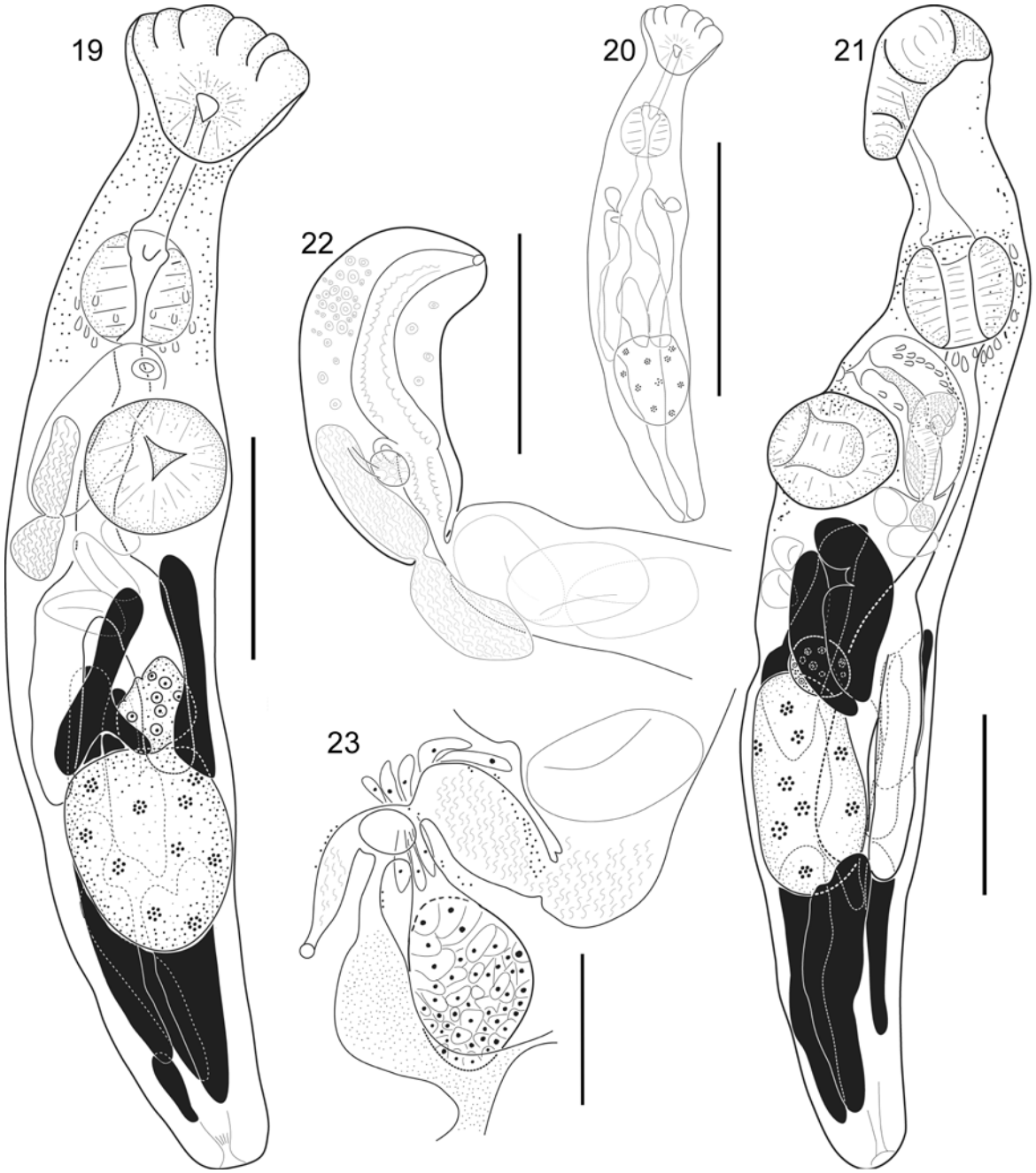
Type-locality: Boat ramp in parking lot of MAGNT, Darwin, Northern Territory (NT), Australia, (12°26'09"S, 130°45'57"E).

Other localities: Buffalo Creek (NT), (12°20'16"S, 130°50'31"E); Sandy Creek (NT), (12°20'37"S, 130°54'05"E).

Site of infection: Intestine.

Type-material: Holotype NTM D001480; paratypes NMT D001481-D001484, USNPC 106219.00-106220.00 and QM G234009, specimens fixed under pressure NMT 001484 and USNPC 106220.00; 2 specimens prepared for SEM; representative DNA sequence of partial 18S, entire ITS region, partial (D1-D3) 28S: GenBank accession no. KC206498, from 6 identical sequences (2 adults from DMANH boat ramp, one adult and one immature specimen from Buffalo Creek, and 2 immature specimens from Sandy Creek).

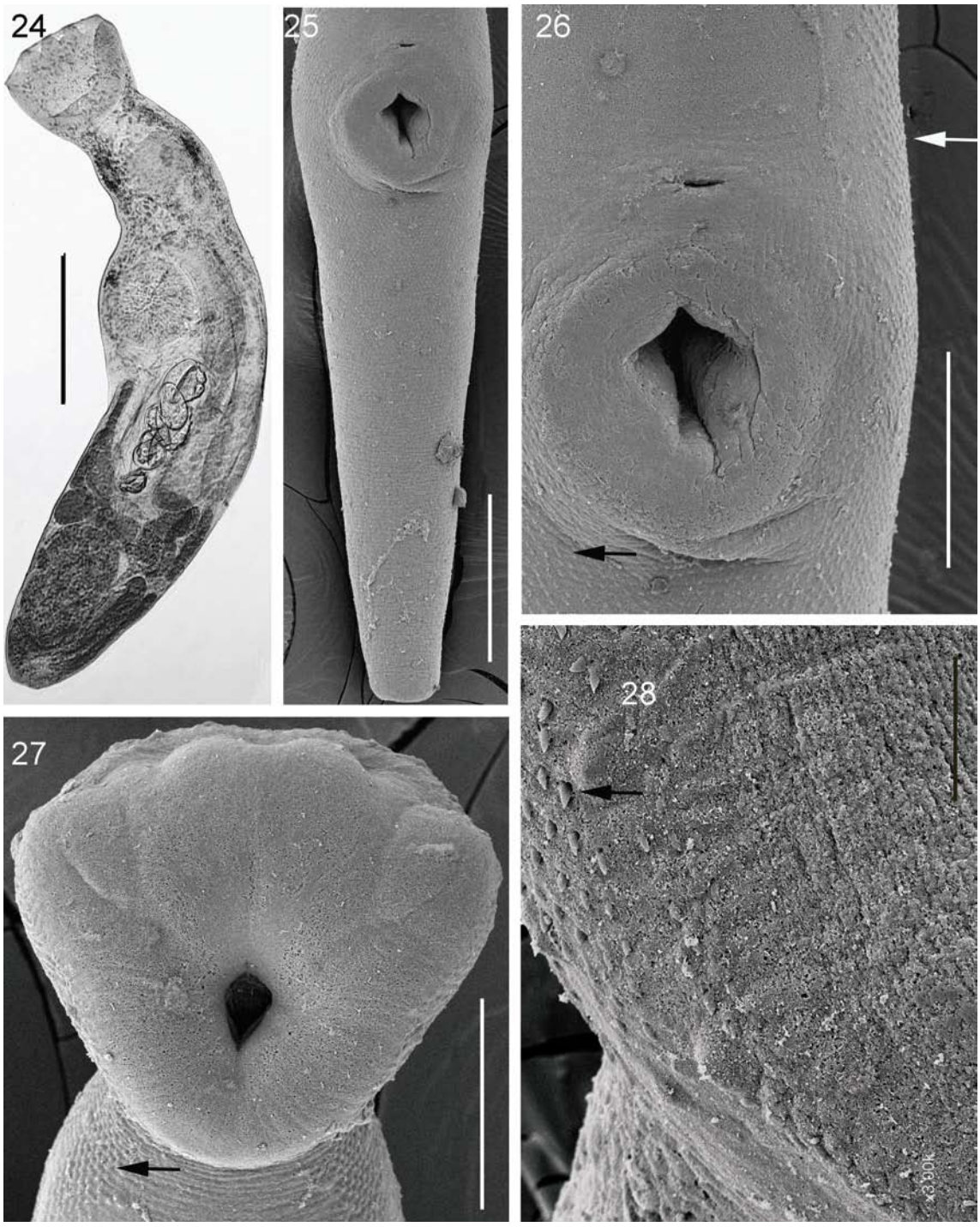
Etymology: The Latinised feminine adjectival name *darwinensis* refers to the Darwin Metropolitan Area, where the holotype and all other specimens were collected.



Figures II.19-23. Capitimitta darwinensis.n. sp. 19. Holotype, ventral aspect of wholemount, not all eggs illustrated; 20. paratype, lateral aspect mount, not all eggs illustrated, 21. holotype, ventral aspect, wholemount showing extent of excretory vesicle; 22. hermaphroditic sac of specimen killed while under pressure; 23. ovarian complex of specimen killed while under pressure. *Scale-bars* 19-21, 200 µm; 22, 100 µm; 23, 50 µm.

Description (Figures 7-8, 19-23, 24-28; Tables 1-4).

Measurements based on 8 gravid, unflattened, wholemount specimens, with those of holotype given in description and of entire series in Table 2. Body long, fusiform, widest near mid-body, 817 long, 148 wide, with width 18% of length. Tegument bears minute spines (Figures 26-28) *c*.2 long; spines densest in region between oral sucker and ventral sucker laterally and dorsally, becoming progressively less dense posteriorly; area of tegument between ventral and oral suckers with few irregularly spaced spines (Figure 26), appearing almost smooth. Eye-spot pigment dispersed in anterior quarter of body. Oral sucker (Figures 7, 19, 20, 27-28) large, V-shaped, terminal, 112 long, 132 wide; 6 papilla-like, muscular structures embedded within sucker, giving scalloped appearance to anterior margin; width of sucker measured at widest point of outer pair of muscular structures considerably wider than body immediately posterior to oral sucker. Mouth opens ventrally. Ventral sucker slightly elevated, 104 long, 98 wide, with anterior margin 283 from anteriormost extremity or 35% of BL; length 88% of oral sucker length; width 79% of oral sucker width. Hindbody 448 or 55% of BL. Prepharynx 96 long, with considerable widening at junction with pharynx; linear length of prepharynx in ventral or dorsal wholemounts shorter than total length because of oblique angle of neck. Pharynx thick-walled, 81 long, 80 wide. Oesophagus150 long, 156% of prepharyngeal length. Glands surrounding prepharynx and pharynx probably associated with digestion. Intestinal bifurcation at level of posterior margin of ventral sucker to slightly further posteriorly, 526 from anterior end of body or 48% of BL. Caeca sac-like, terminate in testicular region, 376 from posterior end of body or 34% of BL.



Figures II. 24-28. Capitimitta darwinensis n. sp. 24. Wholemount of flattened specimen, note displacement of organs to posterior end and appearance of oral sucker; 25-28. scanning electron micrographs: 25. posterior end of body; 26. ventral sucker with spine free patch anteriorly; 27. oral sucker; 28. transition of muscular oral sucker to spinous tegument. Arrows designate spines. *Scale-bars* 24, 200 µm; 25, 100 µm; 26,27, 50 µm; 28, 10 µm.

Testis ovoid, 281 long, 144 wide, 148 from ventral sucker, with post-testicular field 157 long or 19% of body length. External seminal vesicle sac-like, 48 long, 29 wide, with shape variable, often distorted by eggs. Hermaphroditic sac 138 long, 53 wide, with length appearing shorter because of curvature dorsal to ventral sucker, with majority of posterior region dorsal to ventral sucker, contains internal seminal vesicle measuring 65 long by 27 wide, pars prostatica and hermaphroditic duct, with male and female ducts uniting in proximal half. Genital pore medial, anterior to ventral sucker, 262 from anterior end of body or 32% of BL.

Ovary (Figures 19-20) medial, contiguous with testis, 65 long, 50 wide, 88 from ventral sucker, with oviduct arising from anterior portion. Mehlis' gland anterolateral to ovary. Laurer's canal opens dorsally, anterior to or at level of ovary. Vitellarium tubular, commencing 11 from ventral sucker (extending to ventral sucker in most specimens), terminating 58 from posterior extremity of body. Uterus occupies space between hermaphroditic sac and testis, ventral to caeca, with proximal region containing sperm in most specimens, with no sphincter demarcating uterine seminal receptacle. Mature eggs thin-shelled, operculate, 61-64 long, 29 wide (3 measured from holotype), with 11 eggs from 4 specimens fixed under pressure in permanent mounts measuring 61-64 long by 31-39 wide, with terminal eggs not containing miracidium with pigmented eye-spots.

Excretory vesicle Y-shaped, bifurcates at anterior region of testis, posterior to ovary, with arms extending to ventral sucker before reflexing and forming small expansion; pore terminal. Immature specimens generally have same shape as adults. Oral sucker well developed. Caeca relatively wider than in adult, filling predetermined space

of uterus and hermaphroditic sac. Testis well developed. Ovary poorly developed or indistinct; vitellarium not developed.

Capitimitta costata n. sp.

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

Other host: *Scatophagus argus* (Linnaeus), spotted scat (Scatophagidae).

Type-locality: Cabbage Tree Creek, Queensland, Australia, (27°19'47"S, 150°03'11"E) (*Selenotoca multifasciata*).

Other locality: Buffalo Creek, Northern Territory, Australia, (12°20'16"S 130°50'31"E) (*Scatophagus argus*).

Site: Intestine.

Type-material: Holotype QM G234010; paratype, USNPC 106221.00, representative DNA sequence partial 18S, entire ITS region, partial (D1-D3) 28S: GenBank accession no. KC206497, from 3 identical sequences (one adult and one immature specimen from Cabbage Tree Creek, QLD, *Selenotoca multifasciata*, and one adult from Buffalo Creek, NT, *Scatophagus argus*).

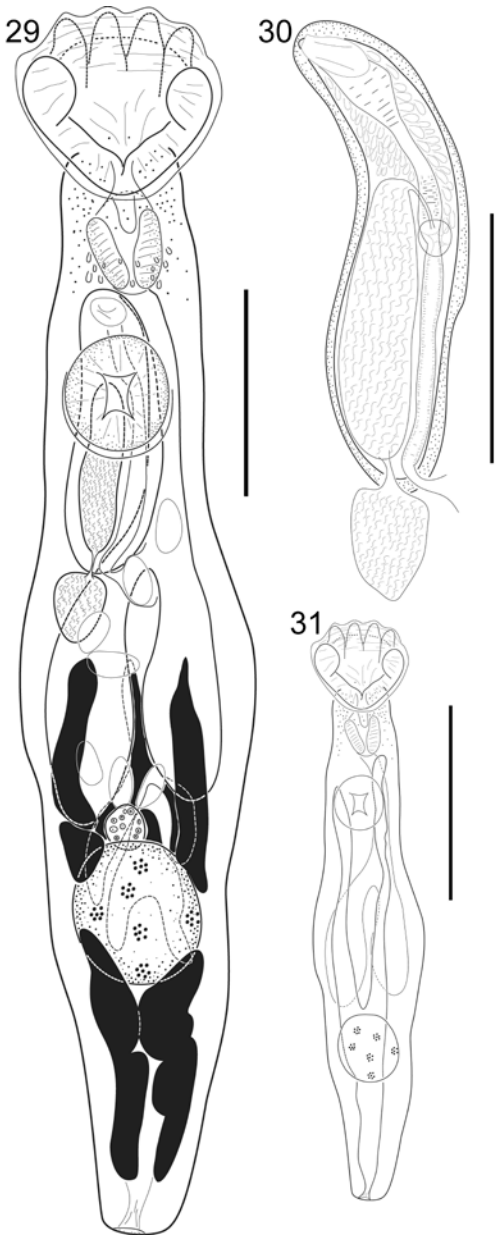
Etymology: The Latin feminine adjective *costata*, meaning "ribbed," refers to the ribbed appearance of the oral sucker resulting from the protrusion of muscular structures.

Description; (Figures 29-31; Table 1-4).

Measurements based on 3 gravid, unflattened, wholemount specimens, with those of holotype given in description and of entire series in Table 2. Body elongate, fusiform, 1,405 long, 268 wide in middle third, with width 19% of BL. Tegument armed with spines; spines 2-4 long, dense except for region between oral and ventral suckers where

only few irregularly spaced spines occur. Eye-spot pigment dispersed in anterior quarter of body. Oral sucker (Figure 29) large, V- to U-shaped depending on amount of expansion, terminal, 207 long, 243, wide, greatly extensible; aperture almost circular; anterior region with 6 muscular, papilla-like structures embedded within sucker, which appear webbed in between giving scalloped appearance to anterior margin of oral sucker; outer pair curved slightly ventrally in holotype, forming slight cup with mouth at base; width, measured at widest point of outer pair of muscular structures, considerably wider than body immediately posterior to oral sucker. Mouth opens ventrally. Ventral sucker slightly elevated, 140 long, 68% of oral sucker length, 127 wide, with width 52% of oral sucker width. Forebody 372 long or 26% of BL; hindbody 911 long or 65% of BL. Prepharynx 35 long, 28% of pharyngeal length, linear length shorter because of position of oral sucker on peduncle. Pharynx 123 long, 92 wide, length 134% of width; prepharynx and pharynx surrounded by dense dispersed eye-spot pigment and gland-cells probably associated with digestion. Oesophagus 287 long. Intestinal bifurcation posterior to ventral sucker or 43% of BL. Caeca sac-like, terminate 492 from posterior end of body or 35% of BL.

Testis ovoid, 183 long, 151 wide, 454 from ventral sucker or 32% of BL; post-testicular space 285 or 20% of BL. External seminal vesicle sac-like, 87 long, 64 wide, variable in shape. Hermaphroditic sac (Figures 29-30) 347 long, 94 wide, thick-walled, terminates well posterior to ventral sucker, contains internal seminal vesicle 207 long by 53 wide, pars prostatica surrounded by prostatic cells, female duct, and hermaphroditic duct; male and female ducts unite roughly at mid-point of hermaphroditic sac; female



Figures II.29-31. Capitimitta costata n. sp. 29. Holotype, ventral aspect mount; 30. holotype, hermaphroditic sac; 31. holotype, ventral aspect, wholemount showing extent of excretory vesicle. *Scale-bars* 29, 250 µm; 30, 200 µm; 31, 500 µm.

duct thin-walled, about half length of sac; hermaphroditic duct highly muscular. Genital pore medial, anterior to ventral sucker, 325 from anterior end or 23% of BL.

Ovary nearly spherical, smooth, near mid-axis of body, slightly dorsal to testis, 56 long, 45 wide, 412 from the ventral sucker. Laurer's canal not observed. Vitellarium

tubular, commences 235 posterior to ventral sucker, terminates 61 from posterior extremity of body. Uterus arises from anterior region of ovary, pretesticular, encroaches into region of ventral sucker, with proximal portion filled with sperm; distal region enters posterior end of hermaphroditic sac, with no evidence of metraterm. Mature eggs thin-shelled, 67-69 long, 27-31 wide (4 measured from holotype), with those in distal uterus not containing miracidium with pigmented eye-spots.

Excretory vesicle Y-shaped, bifurcates near ovary, with arms extending to between levels of ventral sucker and pharynx; excretory pore terminal.

Remarks

Capitimitta costata n. sp. can be distinguished by a number of characters from the slightly smaller *C. darwinensis.* The pharynx of *C. darwinensis* is almost equal in length and width, with its length ranging from 89-108% of its width, whereas in *P. costata*the pharynx is noticeably longer than wide, with the length being 132-134% of its width. The vitelline follicles of *C. darwinensis* commence at between half or less of an ovarian length from the ventral sucker, compared with more than one ovarian length in *P. costata*. The hermaphroditic sac and duct are more muscular and prominent in *C. costata* than in *C. darwinensis*. Finally, the eggs of *P. darwinensis* are shorter and rounder than in *C. costata*, i.e. 58-64 by 29-36 µm rather than 67-70 by 27-31 µm. Both specimens of *C. costata* described from *Selenotoca multifasciata* in Cabbage Tree Creek, Queensland, were fixed with a near maximum expansion of their oral sucker (Figure 29). In the mounted specimen of this species from *Scatophagus argus* at Darwin, NT, the oral sucker appeared the same as that of *C. darwinensis* (Figures 7, 19, 27), with its lobes not extended. rDNA sequences from specimens of *C. costa* from the different hosts in

different localities did not differ, even though the worms were separated by a linear distance of more than 2,800 km, or 3,400 km when calculated around the Cape York Peninsula. The mounted specimen from *S. argus* was slightly contracted but not morphologically different than those from *Selenotoca multifasciata*.

Capitimitta sp.

Host: *Selenotoca multifasciata* (Richardson), spotbanded scat (Scatophagidae).

Locality: Causeway Lake, Queensland, Australia, (23°12'00" S 150°47'21" E).

Site: Intestine.

Material: No mounted specimen; representative DNA sequence, partial 18S, entire ITS region, partial (D1-D3) 28S: GenBank accession no. KC206499, from single immature specimen from Causeway Lake.

Remarks

A single immature specimen was sequenced from *Selenotoca multifasciata* collected at the outlet of Causeway Lake in Queensland, Australia. The sequence obtained from this specimen matched neither the sequence of *C. darwinensis* n. sp. nor *C. costata* n. sp.; therefore, I think it represents an undescribed species. I include it for molecular comparisons and analysis, and encourage others to find and describe it.

Species inquirendae

Waretrema piscicolum of Velasquez (1961) (Figure 9).

This species, reported as *W. piscicolum,* from the scatophagid *Scatophagus argus* in fish ponds at Bulacan and Luzon Island in the Philippines (Velasquez, 1961) appears to belong to *Capitimitta*. I examined three deposited specimens on one slide (USNPC 039476.00), and they conformed with the diagnosis of *Capitimitta*; however, because of

Table II.2

Dimensions and ratios of Capitimitta darwinensis n. sp., C. costata n. sp., Spiritestis herveyensis n. sp. and S. arabii from Red Sea collection (the latter had been killed under varying degrees of coverslip pressure)

Species	*Capitimitta darwinensis*	*Capitimitta costata*	*Spiritestis herveyensis*	*Spiritestis herveyensis* flat	*Spiritestis arabii* GCRL collection
Host	*Selenotoca multifasciata*	*Selenotoca multifasciata, Scatophagus argus*	*Moolgarda seheli*	*Moolgarda seheli*	*Moolgarda seheli, Crenimugil crenilabis*
N	8	2 + 1	6	1	3 + 2
Length	785-1,101 (889)	1,142-1,577 (1,375)	2,491-3,140 (2,936)	3879	2,371-3,249 (2,753)
Width	148-214 (170)	200-287(252)	447-642(551)	840	346-606 (517)
Pre-genital pore distance	226-361(274)	267-339 (310)	643-776 (709)	932	571-785 (660)
Genital pore to ventral sucker	0-75 (31)	8-17 (11)	45-119	185	100-151 (130)
Forebody length	242-388 (307)	372-411(387)	687-898 (803)	1172	710-926 (795)
Hindbody length	411-607 (488)	664-1,036 (870)	1563-1,990 (1,778)	2377	1,389-2,155 (1,710)
Pre-intestinal bifurcation distance	366-526 (425)	573-725 (634)	953-1,220 (1,075)	1470	851-1,165 (1,001)
Postcaecal distance	248-414 (297)	379-528 (466)	518-694 (589)	948	572-887 (706)
Oral sucker (OS) length	110-157 (124)	155-228 (197)	197-277 (253)	303	229-368 (296)
OS width	126-176 (147)	193-255 (230)	279-342 (311)	386	229-397 (328)
Ventral sucker (VS) length	88-126 (103)	108-140 (128)	219-290 (261)	352	212-282 (246)
VS width	98-122(106)	127-155(138)	224-291(258)	371	210-296 (242)
Prepharyngeal length	30-100(72)	35-88(59)	248-396 (342)	443	107-297 (195)
Pharynx length	72-109 (88)	99-123 (113)	157-222 (197)	225	137-209 (172)
Pharynx width	71-102 (85)	75-92 (85)	156-186 (170)	211	147-181 (156)
Oesophagus length	116-240 (181)	287-351(316)	235-365(309)	518	322-482 (381)
Testis length	144-281 (181)	172-217 (191)	457-669(594)	782	713-803 (761)
Testis width	101-150 (119)	135-169 (152)	206-320 (268)	250	215-251 (233)
Testis to ventral sucker	64-202 (124)	258-493 (402)	910-1,184 (1053)	1392	608-1,211 (857)

Table II.2 (continued).

Species	*Capitimitta darwinensis*	*Capitimitta costata*	*Spiritestis herveyensis*	*Spiritestis herveyensis* flat	*Spiritestis arabii* GCRL collection
Post-testicular field	156-280 (194)	235-334 (285)	86-196 (143)	171	42-266 (135)
Ovary width	44-85 (57)	45-69 (55)	140-179 (168)	183	110-162 (134)
Ovary length	101-150 (119)	56-73 (67)	124-199 (153)	199	158-205 (177)
Ovary to ventral sucker	27-167 (79)	195-425 (344)	416-608 (451)	747	413-609 (519)
Testis to ovary	0	0	153-527 (370)	453	16-412 (152)
Vitellarium to ventral sucker	0-36 (6)	98-235 (185)	400-608 (513)	600	243-578 (404)
Vitellarium to posterior end	43-81 (63)	45-61 (52)	36-128 (78)	96	57-110 (72)
Hermaphroditic sac length	99-177 (137)	161-360 (289)	369-510 (429)	611	415-557 (468)
Hermaphroditic sac width	53-81 (67)	75-99 (89)	116-214 (17)	192	163-222 (193)
Internal seminal vesicle length	65-109 (86)	107-227 (180)	181-286 (229)	353	297-434 (357)
Internal seminal vesicle width	27-40 (33)	47-56 (52)	42-88 (71)	58	82-106 (93)
External seminal vesicle length	44-88(63)	69-87 (80)	125-272 (203)	636	444-888 (601)
Externals seminal vesicle width	29-51(38)	48-64 (58)	41-73 (59)	78	85-137 (117)
Clear egg length	58-64 (61)	67-70 (68)	61-67 (64)	54-58 (55)	61-70 (66)
Clear egg width	29-36 (32)	27-31(29)	29-36 (32)	35-40 (38)	33-42 (38)
Width%*	18-21% (19%)	18-19% (18%)	17-24% (19%)	22%	14-26% (19%)
Forebody*	31-38% (34%)	26-33% (29%)	25-31% (29%)	30%	26-31% (29%)
Hindbody*	52-58% (55%)	58-66% (63%)	59-66% (63%)	61%	58-66% (62%)
Pre-intestinal bifurcation distance*	43-53% (48%)	43-50% (46%)	34-41% (38%)	38%	33-41% (36%)
Postcaecal distance*	29-38% (33%)	33-35% (34%)	17-23% (21%)	24%	22-28% (26%)
VS length % OS length	80-88% (84%)	60-70% (66%)	94-111% (104%)	86%	70-104% (84%)
VS width % OS width	67-79% (73%)	52-80% (62%)	76-90% (82%)	82%	59-92% (76%)
Pharynx length % prepharynx length	72-377% (150%)	29-75% (53%)	43-90% (59%)	51%	55-195% (101%)
Pharyngeal length % pharynx width	89-108% (100%)	132-134% (133%)	101-124% (115%)	107%	88-139% (110%)

Table II.2 (continued).

Species	*Capitimitta darwinensis*	*Capitimitta costata*	*Spiritestis herveyensis*	*Spiritestis herveyensis* flat	*Spiritestis arabii* GCRL collection
Testis to ventral sucker*	8-19% (14)	23-32 (29)	34-39% (37%)	36%	26-37% (30%)
Post-testicular space*	15-28% (22)	20-21(21)	3-7% (5%)	4%	2-9% (5%)
Ovary length/ovary to VS distance	0.5-2.6 (1.4)	2.7-7.4 (5.0)	2.8-4.9 (3.5)	3.8	2.4-3.1 (2.9)

***** as percentage of body length

the extreme pressure applied to the specimens when fixed, I cannot with confidence assign its specific status. Eggs measured 60-68 by 23-30, or similar in size to eggs of *C. costata*, but other features prevented us from designating them as conspecific. I think these specimens represent an undescribed species because the arms of the excretory vesicle extend anteriorly to the pharynx, compared with not reaching into the prepharyngeal region as in *C. darwinensis* n. sp. and *C. costata* n. sp. In these Philippine specimens, the oral sucker (Figure 9) does not appear as though it would be as large, proportional to body size, as in *C. darwinensis* (Figures 8, 19) if not flattened, but the lobe-like structures appear embedded, as in the two species I describe. The caeca of the Philippine specimens appear to be less robust than in *C. darwinensis* and *C. costata*, although the flattening of specimens causes a significant shifting of the position of the organs (Figure 24).

Waretrema piscicolum of Liu & Yang (2003)

This species, reported as *W. piscicolum* from the scatophagid *Scatophagus argus* off Zhanjiang, China (South China Sea) (Liu & Yang, 2003), also appears to belong to *Capitimitta*. The thorough description of this worm and the accompanying illustration

(Liu & Yang, 2003) appear to provide an accurate record. The specimens are similar to those of Velasquez (1961) and, although unlikely, may represent the same species. Specimens used by Velasquez are generally larger (1.25-3.15 *vs* 0.96-2.24mm), the oral sucker reported by Liu and Yang appears to be smaller proportionally in relation to the ventral sucker and body size, and the maximum measurement of the ventral sucker diameter is 296 µm in specimens reported by Liu and Yang (2003) and 240 µm in those measured by Velasquez (1961), despite Velasquez's largest specimen being 1.4 times larger than any measured by Lui and Yang.

Waretrema piscicolum of Bilqees (1980)

This species from *Scatophagus argus* off Karachi, Pakistan, was identified as *W. piscicolum* despite clear differences in its oral sucker (Bilqees, 1980; Bilqees, 1981, same figure with additional measurements). Also, the figure showed the intestinal bifurcation located immediately posterior to the pharynx and an indistinct oesophagus. My attempts to borrow the specimens were unsuccessful. I consider this species to represent an undescribed member of *Capitimitta*.

Species incertae sedis

Waretrema piscicolum of Gupta & Miglani (1976)

Although the record of *W. piscicolum* from an unidentified marine fish off Port Blair (Andaman and Nicobar islands), India, by Gupta and Miglani (1976) provides a scant description, their Figure 13 shows a subspherical oral sucker with some type of lobe-like apparatus anteriorly and a pharynx broader than long. These features suggest that this record may represent what I accept as *W. piscicolum*. Although the authors stated that the terminal part of hermaphroditic duct was protrusible, the duct does not protrude

in a properly fixed specimens of members of *Waretrema*, *Spiritestis* or *Capitimitta*; I have seen this condition in other haploporid species when they have been outside the host too long in a non-isotonic solution. Because of the lack of a host identity and questionable state of the described specimens, I consider the material reported by Gupta and Miglani (1976) as *incertae sedis*.

Additional material of species of *Capitimitta* from off China, the Philippines, and Pakistan infecting *Scatophagus argus* and the specimens reported by Gupta and Miglani (1976) from a marine fish in the Andaman and Nicobar islands all require recollection.

Table II.3

Length and number of variable sites based on pairwise comparison of the ITS1 region (above diagonal) and 5.8S gene (below diagonal) between Spiritestis herveyensis n. sp., Capitimitta darwinensis n. sp., C. costata n. sp. and an undescribed species of Capitimitta

		S. herveyensis	*C. darwinensis*	*C. costata*	*Capitimitta* sp.
	Length	*626*	*614*	*449*	*583*
S. herveyensis	*157*	-	206	109	194
C. darwinensis	*157*	4	-	39	68
C. costata	*157*	6	2	-	33
Capitimitta sp.	*157*	3	1	3	-

Table II.4

Length and number of variable sites based on pairwise comparison of the ITS2 region (above diagonal) and partial 28S gene (below diagonal) between Spiritestis herveyensis n. sp., Capitimitta darwinensis n. sp., C. costata n. sp. and an undescribed species of Capitimitta

		S. herveyensis	*C. darwinensis*	*C. costata*	*Capitimitta* sp.
	Length	*310*	*299*	*300*	*301*
S. herveyensis	*1,383*	-	52	58	58
C. darwinensis	*1,370*	147	-	22	33
C. costata	*1,369*	145	38	-	24
Capitimitta sp.	*1,369*	137	37	38	-

Molecular Data

I compared DNA sequence data from three species of *Capitimitta* and *Spiritestis herveyensis*. The fragment sequenced encompassed the 3' end of the 18S gene, the ITS region (ITS1 + 5.8S + ITS2) and *c.*1,300 bp of the 5' end of the 28S gene because this region has been shown to be suitable for species differentiation and phylogenetic analysis (Nolan & Cribb, 2005; Olson & Tkach, 2005; Parker et al., 2010; Tkach et al., 2010). The total length of the region sequenced and used for species discrimination was 2,342 bp in *C. costata*, 2,477 bp in the undescribed *Capitimitta* sp., 2,507 bp in *C. darwinensis* and 2,543 bp in *S. herveyensis*. No intraspecific variation was found in cases where sequences were obtained from multiple individuals of each species. The length differences for different species resulted primarily from indels of various lengths in the ITS1 region (Table 3). For species of *Capitimitta*, the percent variation of the values (Tables 3, 4) were 5.7–11.1% in ITS1, 0.6–1.9% in 5.8S, 7.3–11.0% in ITS2, and 2.7–2.8% in the partial 28S, all of which were consistent with the intrageneric variation found by Blasco-Costa, Balbuena et al. (2009) for species of *Dicrogaster* Looss, 1902 and *Saccocoelium* Looss, 1902. *Capitimitta* spp. differed from *S. herveyensis* (Tables 3, 4) by 17.4– 30% in ITS1, 1.3–3.8% in 5.8S, 14.8 to 17.7% in ITS2 and 10.0–10.6% in the 28S. Levels of intrageneric variation reported by Blasco-Costa, Balbuena et al. (2009) are comparable for the ITS2 and higher for the partial 28S regions for species of haploporines, but lower than those in species that they considered to be in separate subfamilies. My values are consistent with the proposal for the separate generic status for *Capitimitta* and *Spiritestis*.

The BI analysis of partial 28S rDNA gene sequences (Figure 32) included the outgroup *Paragonimus westermani* and two species of the Atractotrematidae in addition

to the 15 species of the Haploporidae. The ingroup of haploporids formed a monophyletic clade. *Hapladena nasonis* appeared to be well-supported as basal to the other haploporids. The other 14 haploporid species formed two clades, one composed of the genera *Dicrogaster*, *Lecithobotrys* Looss, 1902, *Haploporus* Looss, 1902 and *Saccocoelium,* and the other formed by *Forticulcita* Overstreet, 1982, *Spiritestis*, *Saccocoelioides* and *Capitimitta*. *Forticulcita* was basal to a polytomy of the three genera *Spiritestis*, *Saccocoeliodes* and *Capitimitta.* The three species of *Capitimitta* formed a highly supported clade. Because of the high degree of variation among species for available ITS2 sequences, the resulting alignment was highly ambiguous due to indels and left too few informative sites. After the exclusion of ambiguous regions of the alignment, reliable conclusions still could not be made, although the three species of *Capitimitta* formed a clade (data not presented here)

Key to the species of the Haploporidae with an ornamented oral sucker

1a. Vitellarium composed of numerous (>40) small follicles (Figures 10, 12, 14); in Mugilidae ……..... *Spiritestis* Nagaty, 1948 (2)

b. Vitellarium composed of few (*c*.12) large tubular rod-like structures (Figures 19, 20, 24, 29)..... 4

2a. Oral sucker with first pair of oral lobes (ventral pair) directed posteriorly towards oral opening, forming 'W' on the posterior margin of oral sucker (Figures 5, 12); body elongate oval; caeca sac-like.....*Spiritestis machidai* Pulis & Overstreet, 2013

b. Oral sucker with ventral pair of lobes directed anteriorly, with lobes forming 'M'-shape along the anterior rim of the sucker (Figures 2); body elongate; caeca long, relatively narrow 3

3a. Genital pore located at posterior margin of pharynx (Figure 10); from Red Sea .....
.....*S. arabii* Nagaty, 1948.

b. Genital pore at level of pharynx (Figure 14); from Australian waters .....
.....*S. herveyensis* Pulis & Overstreet, 2013.

4a. Oral sucker with six retractable lobes (Figure 1); lobes with spines; infecting Mugilidae .....*Waretrema piscicolum* Srivastava, 1937.

b. Oral sucker with six muscular, non-retractable structures embedded within anterior portion of oral sucker (Figures 7-9, 19-20, 24, 27, 29), lacking spines on ventral surface of sucker (Figures 27-28); infecting Scatophagidae .....
.....*Capitimitta* Pulis & Overstreet, 2013 (5)

5a. Vitelline follicles commencing less than half one ovarian length posterior to ventral sucker (Figures 19-20); pharyngeal length <105% of width; eggs 58-64 × 29-36 µm .....
.....*C. darwinensis* Pulis & Overstreet, 2013

b. Vitelline follicles commencing more than one ovarian length posterior to ventral sucker (Figure 29); pharyngeal length >130% of width; eggs 67-70 × 27- 31 µm .....
.....*C. costata* Pulis & Overstreet, 2013

Discussion

The status of the type-species of *Waretrema* is uncertain, as discussed above, and the only possible valid report, other than the original description, is by Gupta and Miglani (1976). They reported the host as a "marine fish," and illustrated the broad pharynx and nature of the oral sucker similar to that originally illustrated for *W. piscicolum* by Srivastava (1939). The specimens of Machida (1996) attributed to *W. piscicolum* clearly represented a species of *Spiritestis* based on the oral sucker, pyriform pharynx and

vitellarium with numerous follicles. Despite the contracted state of the specimens described by Nagaty (1948) as *S. arabii*, and with the addition of the Red Sea specimens also from mullet, the general nature of the oral sucker, pharynx, and vitellarium shows this species to be considerably different from those which I have placed in *Capitimitta* n. g. The other three records of *W. piscicolum* were reported as hosted by scatophagids. Molecular data from *S. herveyensis* n. sp. support the decision to resurrect *Spiritestis*. The reports from scatophagids by Velasquez (1961), Bilqees (1980) and Liu and Yang (2003) all involve material that has the general characteristics of *Capitimitta*, and most likely include undescribed species. The absence of subsequent records of species of *Waretrema*-like haploporids from other locations and from mullets is perplexing. The illustration and

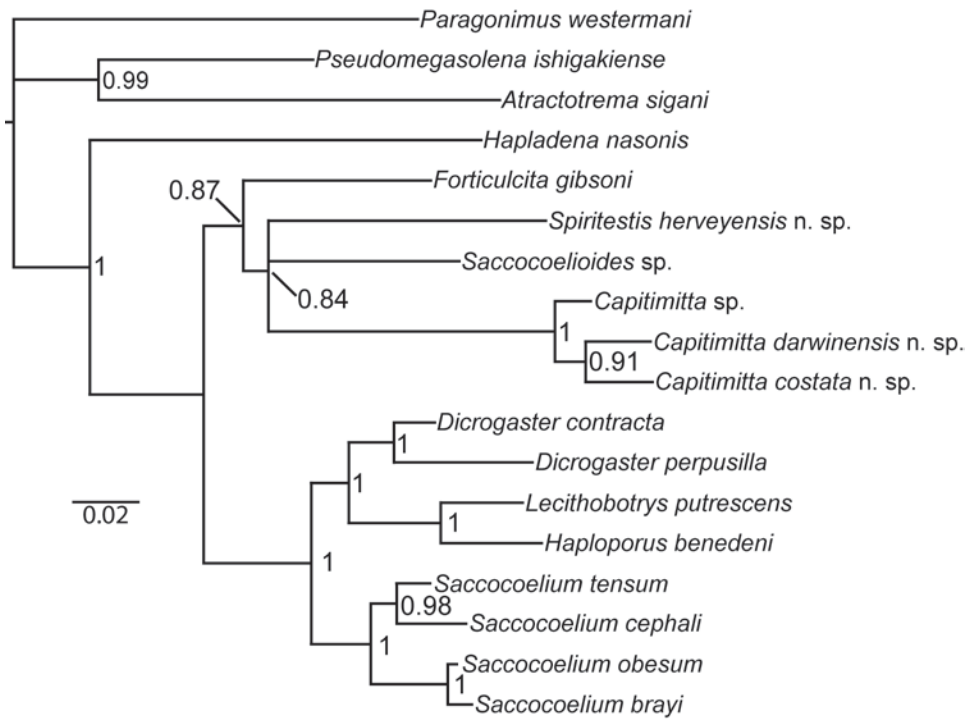


Figure II.32. Estimated position of *Spiritestis* and *Capitimitta* with in the haploporidae. Phylogenetic relationships of the Haploporidae using partial 28S rDNA sequences with *Paragonimus westermani* as the outgroup. Posterior probability score given at the nodes (see Table 1 for accession numbers).

description by Srivastava (1939) show an almost *Capitimitta*-like oral sucker, and the of the vitellarium and pharynx are similar. In subsequent reports, no species referable to *Waretrema* or *Capitimitta* has been reported from a mullet.

Species of *Spiritestis* have been reported from the mullets *Moolgarda seheli* and *Crenimugil crenilabis*. Specimens morphologically identified as *M. seheli, C. crenilabis,* unidentified specimens of *Moolgarda* spp. and *Valamugil* spp. from many areas have been found to form a monophyletic clade, probably comprising five species (Durand, Shen et al., 2012). Specimens identifiable as *M. seheli* from Australian waters (host to *S. herveyensis* n. sp.) belonged in the same clade, but were distinct from the other members identifiable as *M. seheli* from the other areas and identified as *C. crenilabis*. This distinctness in hosts further supports a difference in the species of *Spiritestis*. Based on their hosts, species of *Spiritestis* appear more closely related to species of *Waretrema* than to species of *Capitimitta*. Moreover, I also collected specimens of the more distantly related *Liza vaigiensis* from the waters of Western Australia, Northern Territory, and Queensland and found no species of haploporid with ornate muscularisation in the region of the oral sucker.

All specimens of *Capitimitta* I collected occurred in the scatophagids *Selenotoca multifasciata* and *Scatophagus argus* measuring under 12 cm, and each infected fish harboured only a few individuals. Only nine specimens of *Selenotoca multifasciata* and no specimen of *Scatophagus argus* larger than 12 cm were examined, but many specimens of *Selenotoca multifasciata* between 12 and 24 cm, in some cases even caught in the same throw of the castnet as the small infected individuals, harboured numerous trematodes other than members of *Capitimitta*. Known life-cycles of waretrematines

involve the ingestion of cercariae (Sheena & Janardanan, 2007) or metacercariae (Tang & Lin, 1979; Shameem & Madhavi, 1991;Diaz et al., 2009). As the life-cycle for a species of *Capitimitta* is unknown, I can only speculate that the pattern involves diet change by the host related to growth or competition among these and other trematode species; regardless, species of *Capitimitta* seem to be replaced with other trematodes in large fish. The gut contents of small *S. multifasciata* consisted mostly of filamentous algae, and those of large individuals consisted of leaf-like matter (EEP, pers. obs.). No report of material that I consider belonging to *Capitimitta* in scatophagids (Velasquez, 1961; Bilqees, 1980; Liu & Yang, 2003) indicated host sizes, but perhaps exhaustive sampling of large fish would produce infections of *Capitimitta*. Future life-cycle work involving the first intermediate host of a species of *Capitimitta* should begin with snails of the Rissooidae and Potamididae, families found to host other haploporids, that inhabit filamentous algae and mangrove habitats in areas where species of *Capitimitta* have been found in scatophagids.

I used a partial 28S alignment of the three species of *Capitimitta,* including the unnamed species represented by juveniles, and *Spiritestis herveyensis*, all waretrematines, along with some available haploporid sequences from GenBank (Table 1). *Hapladena nasonis* appears basal in the haploporid clade, in agreement with Blasco-Costa, Balbuena et al. (2009). Blasco-Costa, Balbuena et al. (2009) proposed the new subfamily Forticulcitinae for *Forticulcita,* because of the paraphyletic classification of the haploporines if *Forticulcita* is included. I agree that *Forticulcita* does not belong to the subfamily Haploporinae, but, because of the paucity of genera available for molecular

analysis, there remains considerable uncertainty whether the subfamily framework of Overstreet and Curran (2005) is valid or an artificial arrangement.

The result of incorporating sequence data for *S. herveyensis* and *Capitimitta* spp. with previously published sequences and analyses by BI (Figure 32), shows that *Capitimitta* and *Spiritestis* are no more related to each other than they are to *Saccocoelioides*; consequently, I could consider both *Forticulcita* and *Saccocoelioides* as members of Waretrematinae or I could propose a distinct subfamily for either *Spiritestis* or *Capitimitta*, although none of these proposals seem warranted until more genera of the Haploporidae are analysed. I view the morphologically-based subfamilial placements proposed by Overstreet and Curran (2005) with scepticism because molecular data are now available for eight of about 29 genera but for only two type-genera, and these do not emphasise geography and convergent evolution. From a geographical and morphological standpoint, the close grouping of *Dicrogaster*, *Lecithobotrys*, *Haploporus*, and *Saccocoelium* seems logical, and all fit well into the Haploporinae. The monophyly of this portion of the clade may result from diversification within the Mediterranean Sea, but other currently accepted haploporines may not fit well with this group. The absence of available species from areas outside the Mediterranean Sea, and of other genera placed within the Haploporinae, may represent a monophyly of portions of the clade resulting from diversification within the Mediterranean Sea and not accounting for the diversity of species currently regarded as haploporines according to Overstreet and Curran (2005). Until more type-species and representative haploporid and haploporid-like species are sequenced and analysed, any major revision or change should proceed with caution. At the current time, five haploporid subfamilies are recognised, but sequence data for the

type-genera exist for only *Forticulcita* and *Haploporus.* I emphasise this uncertainty by separating the waretrematine species of *Capitimitta*, *Spiritestis*, and *Waretrema*. Based on published records, descriptions, and systematic treatments, I would have incorrectly expected species attributed to *Spiritestis* and *Capitimitta* to be closely related. The appearance of the superficial similarity of the oral sucker is not the result of shared evolutionary history; rather, the ornate muscularisation seems to be acquired at least twice. Also, I do not think *W. piscicolum* has been convincingly reported since the original description by Srivastava (1937, 1939), and *W. piscicolum* (*sensu stricto*) may not be closely related to either *Spiritestis* or *Capitimitta*. Using either *Spiritestis* or *Capitimitta* as a surrogate in phylogenetic treatment of the subfamily is not advisable, as one or both genera may not be members of the Waretrematinae. *Waretrema* shares more characteristics in common with *Capitimitta* than with *Spiritestis*, but the affiliation with the definitive hosts would suggest a closer relationship with *Spiritestis*.

The present study demonstrates the poorly-understood nature of the species diversity of haploporids and the importance of proper methods for fixing and preserving trematodes. For reliable morphological differentiation of haploporid genera and species, specimens for comparison should be killed with hot steaming, but not boiling, water without pressure. Other hot fixatives also produce good results but are more noxious than hot tap water. With species that possess an ornamented oral sucker, killing with pressure precludes full appreciation of the nature of the oral apparatus (Figures 8-9, 10, 12, 24) and leads to erroneous interpretations of those features, as well as causing shifts in the position of some internal organs (Figure 24). However, pressure applied to a few additional specimens not used for measurements or the precise location of their organs

allows the most critical interpretation of the terminal genitalia and the female complex. For example, problems arising from not using hot fixatives and the improper handling of specimens has led to several misidentifications of species as *W. piscicolum*. Molecular data should be collected whenever possible to accompany fixed and mounted specimens and to produce phylogenies.

CHAPTER III

A NEW SPECIES OF *INTROMUGIL* (DIGENEA: HAPLOPORIDAE) AND REDESCRIPTION OF *INTROMUGIL MUGILICOLUS*

Abstract

Intromugil alachuaensis n. sp. is described based on specimens collected from the flathead grey mullet (*Mugil cephalus*) from the Santa Fe River in Florida. The new species is the fourth recognized species in the genus and the second from North America, with the other two being confined to South America. *Intromugil mugilicolus* from Louisiana and Mississippi is redescribed based on the holotype and newly collected material that was not flattened prior to fixation. Two generic features not previously reported are apparent in the new material from *I. mugilicolus* and *I. alachuaensis* n. sp.: an armed oral sucker and a series of sacs containing glandular material arranged in symmetrical rows in the hermaphroditic duct. *Intromugil alachuaensis* differs from *I. mugilicolus* by having an oral sucker longer than wide, body spines smaller and lanceolate rather than longer and hastate, and smaller vitelline follicles. *Intromugil alachuaensis* n. sp. differs from *Intromugil simonei* by having a large elongated pharynx rather than a small subspherical one, a proportionally larger and longer oral sucker, and a longer prepharynx (greater than one pharyngeal length). *Intromugil alachuaensis* n. sp. differs from *Intromugil annakohnae* by having a longer than wide pharynx, a relatively large oral sucker, less extensive vitellarium, and smaller body spines. Comparison of more than 2,400 base-pair long sequences of nuclear rDNA (partial 18S, complete ITS1, complete 5.8S, complete ITS2, and partial 28S) from *I. mugilicolus* and *I. alachuaensis* n.

sp. reveals 110 pairwise differences, including gaps, thus supporting my proposal of a new species. These represent the first published sequences from species in this genus.

Introduction

During examinations of flathead grey mullet (*Mugil cephalus* Linnaeus, 1758) collected from Davis Bayou, Mississippi, and the Santa Fe River, Florida, for helminths, I encountered two species of *Intromugil* Overstreet and Curran, 2005, one of which represented an undescribed species. *Intromugil* was erected by Overstreet and Curran (2005) to accommodate *Carassotrema mugilicolum* Shireman, 1964, as the type-species for the genus. Overstreet (1971) had previously transferred *Carassotrema mugilicolum* to *Chalcinotrema* Freitas, 1947. *Chalcinotrema simonei* Travassos, Freitas, and Bührnheim, 1965 also was assigned to *Intromugil* (see Overstreet & Curran, 2005). The subsequent description of *I. annakohnae* Fernandes and Cohen, 2006 brought the total number of species reported for the genus to three. All three original descriptions featured material that had been killed under coverslip pressure, a method that appears to have shifted the position and shape of some organs, thus altering some species-level diagnostic features. *Intromugil mugilicolus* has been reported from *M. cephalus* in Louisiana, USA (Shireman, 1964). Also, Conroy and Conroy (1986) reported *I. mugilicolus* from the white mullet (*Mugil curema* Valenciennes, 1836) in Falcon, Venezuela; however, I suspect that the identification should have been *I. annakohnae*. *Intromugil simonei* was described from the Lebranche mullet (*Mugil liza* Valenciennes, 1936 reported as *Mugil platanus* Günther, 1880) in Espirito Santo, Brazil (Travassos et al., 1965), and supplemental material was described from *M. curema* in Sao Paulo, Brazil (Conroy & Conroy, 1984). *Intromugil annakohnae* was described from *M. liza* in Rio de Janeiro

State, Brazil by Fernandes and Cohen (2006). In the present study, I redescribe *I. mugilicolus* using the holotype plus supplemental specimens and describe the new species of *Intromugil* from *M. cephalus* in Florida. In addition, I present ribosomal DNA (rDNA) sequence data for *I. mugilicolus* and the new species and compare divergence between the two.

Materials and Methods

Mugil cephalus was collected from 2007 through 2012 from the Gulf Coast Research Laboratory pier, Davis Bayou, Mississippi, (30°23'30"N, 88°47'57"W) with a cast net and in 2011 from the Santa Fe River, Florida (between points 29°51'54"N, 82°44'24"W 29°50'27"N, 82°42'20"W) with a Hawaiian sling. Fish length was measured as total length (TL), extending from the tip of the snout to the end of the tail. Fish names follow those in FishBase (Froese & Pauly, 2013). Live worms from the fish were rinsed in saline, examined briefly, killed with hot steaming water (not boiling), and fixed in 70% ethanol. Fixed worms were stained in aqueous alum carmine, Mayer's hematoxylin, or Van Cleave's hematoxylin, dehydrated in a graded ethanol series, cleared in clove oil (carmine and Van Cleave's) or methyl salicylate (Mayer's), and mounted permanently in Damar Gum. For comparisons, some specimens also were killed under coverslip pressure and fixed in 70% ethanol. Some living worms were examined in detail with a compound microscope. Measurements of fixed material were taken with a differential interference contrast (DIC) equipped compound microscope using a ProgRes® CapturePro camera (ver. 2.8 Jenoptic, Jena, Germany) and software. All measurements are in micrometres (µm) unless otherwise noted.

The holotype, of *I. mugilicolus* from the United States National Parasite Collection (USNPC No. 6006-5), labeled as *Carassotrema mugilicolum*, with an additional label from 2003 indicating *Intromugil* n. g. by Overstreet and Curran, was examined, for comparison with new material. Specimens were deposited in the USNPC Beltsville, Maryland and in the Gulf Coast Research Laboratory Museum, Ocean Springs, Mississippi (GCRLM).

Genomic DNA was extracted from individual specimens of *I. mugilicolus* (n = 4) and the new species (n = 2) using Qiagen DNAeasy tissue kit (Qiagen, Inc., Valencia, California) following the instructions provided. DNA fragments of approximately 2,500 base pairs (bp) comprising the 3' of the 18S nuclear rDNA gene, internal transcribed spacer region (ITS) (= ITS1 + 5.8S + ITS2), and the 5' end of the 28S gene (including variable domains D1-D3) were amplified from the extracted DNA by polymerase chain reaction (PCR). Forward primer ITSF (5' - CGCCCGTCGCTACTACCGATTG-3') and reverse primer 1500R (5'-GCTATCCTGAGGGAAACTTCG-3') were used for PCR reactions. The same PCR primers and multiple internal primers were used in sequencing reactions, the internal forward primers were digl2 (5'-AAGCATATCACTAAGCGG-3'), 300F (5'-CAAGTACCGTGAGGGAAAGTTG-3'), 900F (5'-CCGTCTTGAAACACGGACCAAG-3'); internal reverse primers were Digl2R (5'-CCGCTTAGTGATATGCTT-3'), 300R (5'-CAACTTTCCCTCACGGTACTTG-3'), and ECD2 (5'-CTTGGTCCGTGTTTCAAGACGGG-3').

The resulting PCR products were purified with Qiagen Qiaquick™ columns, cycle-sequenced using ABI BigDye ™ chemistry (Applied Biosystems, Inc., Carlsbad, California), alcohol-precipitated, and run on an ABI 3130 Genetic Analyzer™.

Contiguous sequences were assembled using Sequencher™ (GeneCodes Corp., Ann Arbor, Michigan, 5.10) and submitted to GenBank. Sequences were aligned using ClustalW application in BioEdit 7.0.9 (Tom Hall, Ibis Biosciences, Carlsbad, California [2007]). The limits among the 5.8S, ITS2, and 28S gene fragments were located using the Internal Transcribed Spacer 2 Ribosomal Database (Keller et al., 2009). Presence of variation across replicates and species was looked for in the regions sequenced.

Results

Intromugil mugilicolus (Shireman, 1964) Overstreet and Curran, 2005

Syns Carassotrema mugilicola Shireman, 1964; *Chalcinotrema mugilicola* of Overstreet (1971); *Intromugil mugilicolus* of Overstreet and Curran (2005)

Taxonomic summary

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

Site: Intestine.

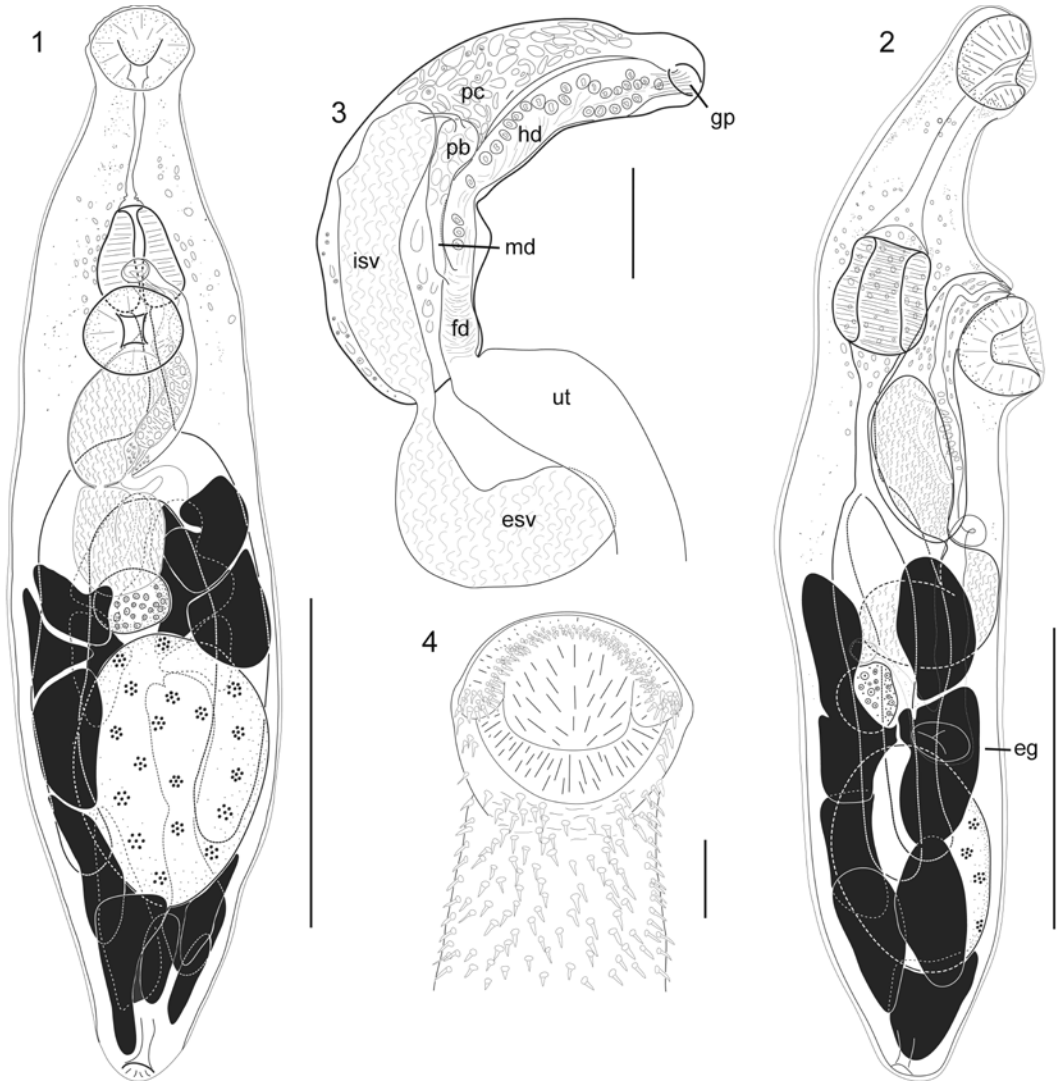
Type-Locality: Brackish pond, Norco, St. Charles Parish, Louisiana.

Locality of additional material: Davis Bayou, Ocean Springs, Jackson County, Mississippi (30°23'30"N, 88°47'57"W).

Specimens deposited: Holotype, USNPC 6006-5; voucher material USNPC 106385.00 - 106386.00, GCRLM 06356-06358.

Representative sequence deposited: partial 18S, entire ITS region, partial (D1-D3) 28S: GenBank accession no. KC430096 (identical sequences from 4 individual worms).

Redescription: (Figures 1-4, 9; Tables 1,2).



Figures III.1-4. Intromugil mugilicolus (Shireman, 1964) from *Mugil cephalus*. 1. Ventral view of nonovigerous wholemount, scale bar = 600 µm (USNPC 6006-5). 2. Lateral view of wholemount, only dextral vitelline follicles illustrated, eg = egg, scale bar = 600 µm. 3. Hermaphroditic sac of holotype (USNPC 6006-5); specimen had been killed under coverslip pressure, and not all glands in hermaphroditic sac clearly visible, esv = external seminal vesicle, fd = female duct, gp = genital pore, hd = hermaphroditic duct, isv = internal seminal vesicle, md = male duct, pb = prostatic bulb, pc = prostatic cells, ut = uterus, scale bar = 100 µm. 4. Oral sucker showing 3 rows of diminutive spines along anterior margin of mouth and tegumental spines, scale bar = 50µm.

Based on holotype, two newly collected flattened specimens, and 10 unflattened wholemounts (6 mounted ventrally and 4 laterally), with measurements in description based on illustrated wholemount (Figure 1) unless otherwise noted; non- flattened

specimens represented in Table I. Body elongate, tapered at both ends, 2,106 long, 530 wide, with width measured in hindbody where widest, with width 25% of body length BL, whitish in life. Tegument spinous over entire surface; spines becoming smaller and more sparse posteriorly; spines measured from flattened specimens in area anterior to ventral sucker on ventral surface ensuring proper orientation, with very slight bend towards tegument when viewed laterally, 15-20 long, hastate, with ovoid root, 5-7wide by 3-5 long, with blade 10-13 long and pointed tip. Eye-spot pigment dispersed throughout body, most prominent in anterior half of body. Oral sucker subterminal, opening ventrally, 159 long, 188 wide, armed with 3 rows of spines along anteriormost margin of mouth; spines diminutive and peg-like in middle oral sucker, with spines in lateral corners much larger than those in central oral sucker; approaching the size of tegument spines, with about 35-38 spines per row (Figure 4); spines apparently missing or worn off in some specimens, with lateral corners slightly elevated forming widest portion and appearing lobe-like. Ventral sucker slightly elevated, 175 long, 199 wide, covered by small blunt concentric rows of spines; similar in size and shape to other spines on body. Oral sucker length 91% of ventral sucker length, with width 94% of ventral sucker width. Forebody 563 or 27% of BL; hindbody 1,369 or 65% of BL. Prepharynx well-defined, 232 long, surrounded by numerous gland cells, with posterior portion of oral sucker surrounded by densest concentration of eye-spot pigment. Pharynx pyriform, thick-walled, 219 long, 165 wide, with length 133% of width and length 94% of prepharyngeal length oesophagus less distinct than prepharynx, 240 long. Intestinal bifurcation near posterior margin of ventral sucker, 869 from anterior end or 41% of BL, caeca terminating 464 from posterior end of body or 22% of BL.

Testis medial, intercaecal, 550 long, 371 wide, 498 from ventral sucker or 24% of BL; post-testicular space 341 or 16% of BL. Hermaphroditic sac arcuate, passing dorsal to ventral sucker, often slightly displacing esophagus, 456 long, 186 wide; sac containing following structures (internal seminal vesicle 283 long by 124 wide, male duct thin-walled; prostatic bulb situated laterally to anterior half of internal seminal vesicle; female duct joining pars prostatica in posterior third of sac as hermaphroditic duct); hermaphroditic duct strongly muscularized for about 3/4 length of hermaphroditic sac, containing a series of 42-46 sacs bearing glandular material; sacs in 2 symmetrical rows. Genital pore medial, anterior to ventral sucker, 506 from anterior end of body or 24% of BL. External seminal vesicle sac-like, 227 long, 176 wide, often folded over on itself and displaced by uterus.

Ovary subspherical, medial, slightly anterior or contiguous with anterior dorsal edge of testis, 135 long, 150 wide, 354 from ventral sucker, with oviduct exiting from anterior portion. Mehlis' gland anterior to ovary. Laurer's canal present; opening not observed. True seminal vesicle absent. Vitellarium with elongate rod to plate-like follicles, with largest follicles roughly 550 long; follicles numbering about 15 or fewer, surrounding testis laterally and dorsally, confluent in post-testicular space, extending anteriorly to 203 from ventral sucker, terminating 53 from posterior end. Uterus looping posterior to anterior level of testis in ventral hindbody before ascending to hermaphroditic sac, with proximal portion filled with sperm. Eggs thin-shelled, operculate, numbering 1 to 63 (mean 14.2 from 12 ovigerous specimens), slightly golden, 67-73 long, 38-47 wide when measured from the distal portion of uterus. Distal eggs containing underdeveloped miracidia lacking eye-spots; miracidium in eggs cultured for

7 days in saline developing eye-spots before degenerating; proximal eggs recently formed, slightly larger. Lymphatic system prominent in living material. Excretory vesicle I-shaped, widest anteriorly, ascending to level of ovary, distinct in live but not fixed specimens, usually collapsing within few minutes in saline outside of host; pore terminal.

Remarks

My flattened specimens were morphologically consistent with the holotype. Shireman (1964) did not report the number of specimens measured in the original description, but I presume it was small because his measurements had a much smaller range of variability compared with that of my material. My observations revealed 2 previously undetected taxonomically important features: first, 3 rows of diminutive spines on the anterior margin of the mouth were apparent in specimens killed without coverslip pressure. The oral spines were relatively smaller than the tegumental spines (See Figure 4), peg-like centrally, becoming larger, and approaching the shape and size of the tegumental spines laterally; these were discernable only in worms that had been killed without coverslip pressure. Even in the flattened specimens, I could not definitively discern if the oral spines were originating from the oral sucker or in the edge of the tegument where it joins the oral sucker. The posterior half of the oral sucker was smooth. Second, in the hermaphroditic duct, which did not contain scleritization, there was a series of sacs containing a glandular substance, whose function requires further investigation. In both living specimens and those heat-killed and stored in ethanol, the sacs were readily discernable when examined using dissecting scope or a DIC-equipped compound scope. After processing the worms for permanent mounts, I could no longer observe the structures easily. Also, fixation under coverslip pressure displaces organs,

potentially leading to erroneous conclusions. For example, pressure on *I. mugilicolus* displaces the testis posteriorly, but without pressure the caeca terminate at the level of the posterior half of the testis rather than at the anterior margin, and the vitellarium converges in the post-testicular space. In flattened specimens, the Mehlis' gland often occurs posterior to the ovary, whereas in unflattened ones it occurs anterior or lateral to the ovary. In addition, flattening made the oral sucker appear terminal, whereas the oral sucker was subterminal with the mouth opening ventrally in material both living material and that fixed without pressure.

Intromugil mugilicolus exhibits the following features that serve to distinguish it from congeners: (1) the prepharynx is considerably longer than in *I. simonei* and *I. annakohnae*, (2) the pharynx is relatively large and elongate compared with the small one in *I. annakohnae* and the spherical one in *I. simonei*, (3) the esophageal length is about 1 pharynx length compared with about 3 pharyngeal lengths in *I. simonei*, (4) although, not reported for *I. simonei*, the tegumental spines of *I. annakohnae* are small relative to those in *I. mugilicolus*, (5) vitelline follicles of *I. mugilicolus* are much larger than those in *I. simonei*, and (6) eggs are shorter than those in *I. annakohnae* or in *I. simonei*.

Intromugil alachuaensis n. sp.

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

Site: Intestine.

Prevalence: 6 of 8 infected.

Type- Locality: Sante Fe River, where forms border between Columbia and Gilchrist counties, Florida, USA, between points 29°51'54"N, 82°44'24"W and 29°50'27"N, 82°42'20"W.

Specimens deposited: Holotype, USNPC 106387.00; paratypes USNPC106388.00, GCRL 06359-06360.

Representative sequence deposited: partial 18S, entire ITS region, partial (D1-D3) 28S: GenBank accession no. KC430095 (Identical sequences from 2 individual worms).

Etymology: The Latinized masculine name *alachuaensis* refers to 'Alachua' or 'chua', which are Timucuan for 'sinkhole', alluding to the geological nature of the springs that were the source of the river and the infected fish.

Description: (Figures 5-8, 10; Tables I-II)

Based on 6 heat-killed specimens without pressure, 2 flattened specimens, and 2 specimens used for molecular sequencing. Measurements of holotype given in text, with those of unflattened specimens given in Table I. Body elongate, fusiform, 2,178 long, 553 wide, widest in hindbody, with width 24% of BL, dark yellowish brown in life. Tegument spinous over entire surface; spines becoming smaller and more sparse posteriorly, measured from flattened specimens, 9-13.5 total length, lanceolate, triangular when viewed laterally, with root 4-6 wide by 1.5-3 long, with blade 6-11 long when measured in area anterior to ventral sucker on ventral surface. Eye-spot pigment most prominent in anterior half of body. Oral sucker infundibuliform, subterminal, opening anteroventrally, 312 long, 276 wide, armed with 3 rows of spines along anteriormost margin of mouth, spines diminutive and peg-like in middle oral sucker, with spines in lateral corners much larger than those in central oral sucker approaching the size of tegument spines; spines numbering 45-55 in each row a few not attributable to particular row near lateral ends of rows. Ventral sucker slightly elevated, nearly spherical, 164 long, 208 wide, appearing ovoid when not orientated parallel to rest of body, covered by concentric rows of spines;

sucker spines similar in shape to body spines, smaller than body spines; oral sucker length 190% of ventral sucker length, width 133% of ventral sucker width. Forebody 754 or 35% of BL; hindbody 1,257 or 58% of BL. Prepharynx well defined, 245 long. Pharynx thick-walled, muscular, with longitudinal edges nearly parallel, 242 long, 167 wide, with length 145% of width; pharynx and prepharynx surrounded by numerous gland cells, surrounded by densest concentration of eye-spot pigment. Oesophagus less distinct than prepharynx, 210 long. caeca terminating 282 from posterior end of BL; postcaecal space 22% of BL; bifurcation in region of ventral sucker, 1,044 from anterior end or 48% of BL.

Testis medial, intercaecal, 440 long, 260 wide, 624 from ventral sucker or 29% of BL; post-testicular space 222, representing 10% of BL. Hermaphroditic sac situated dorsal to ventral sucker, displaced from medial, 314 long, 155 wide, terminating posterior to ventral sucker; sac containing the following structures (internal seminal vesicle, 202 long by 97 wide; male duct thin-walled; prostatic bulb prominent with size dependent on amount of sperm and eggs present; male duct joining female duct in posterior half of sac); hermaphroditic duct strongly muscularized, about half length of sac, containing series of 18 sacs bearing glandular material; sacs arranged in 2 symmetrical rows, dark grey to blackish in color in life. External seminal vesicle sac-like, 150 long by 129 wide, often obscured by eggs. Genital atrium small, hermaphroditic sac appearing as several diverticula, without evidence of scleritization; genital pore immediately anterior to ventral sucker, medial, 714 from anterior end or 33% of BL.

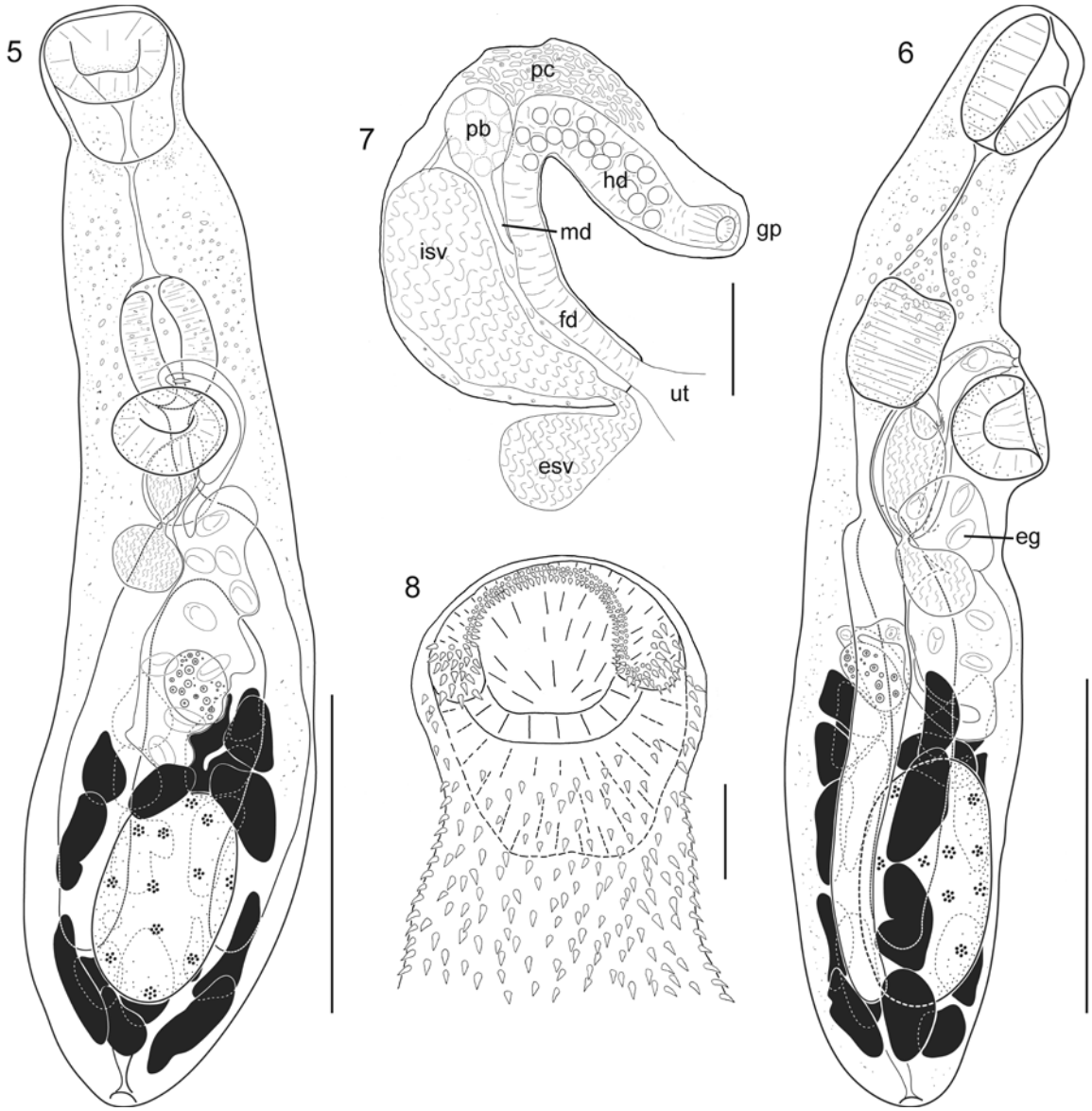
Ovary medial, pretesticular, intercaecal, 123 long, 114 wide, 394 from ventral sucker, 134 from testis, with oviduct emerging from anterior portion. Mehlis' gland

anterior to ovary. Laurer's canal not observed. True seminal receptacle absent. Vitellarium consisting of about 20 or fewer ovoid follicles, usually not contiguous with one another, about 150-275 long, surrounding testis, confluent in post-testicular space, extending anteriorly to 440 from ventral sucker, terminating 70 from posterior end or 3% of BL. Uterus descending to testis then ascending to hermaphroditic sac, with proximal portion filled with sperm; metraterm lacking. Eggs thin-shelled, operculate, 2-47 in number (mean 23.5, from 12 ovigerous specimens), 71-86 long, 38-43 wide when measured from distal portion of uterus, with underdeveloped miracidium lacking eye-spots; proximal eggs slightly larger. Lymphatic system prominent in living material. Excretory vesicle I-shaped, elongate, dorsal to testis, extending to near ovary, increasing in width anteriorly; pore terminal.

Remarks

Based on a combination of morphological features, including spined tegument, single testis, and hermaphroditic sac, the new species belongs in the family Haploporidae Nicoll, 1914. The elongate body, long cylindrical caeca terminating in the testicular region, absence of pigmented eye-spots in miracidium, location of uterus confined between the testis and ventral sucker, long arcuate hermaphroditic sac, and American mugilid host place the species in *Intromugil*.

Intromugil alachuaensis n. sp. can be easily differentiated from the previously described congeners based on a number of morphological features: (1) the infundibuliform, elongated oral sucker that differs from the wider oral sucker in the other three species of



Figures III.5-8. Intromugil alachuaensis n. sp. from *Mugil cephalus*. 5. Ventral view of holotype, scale bar = 600 µm. 6. Lateral view of paratype, scale bar = 600 µm. 7. Hermaphroditic sac of specimen killed under coverslip pressure; esv = external seminal vesicle, fd = female duct, gp = genital pore, hd = hermaphroditic duct, isv = internal seminal vesicle, md = male duct, pb = prostatic bulb, pc = prostatic cells, ut = uterus, scale bar = 100 µm. 8. Oral sucker showing 3 rows of diminutive spines along anterior margin of mouth and tegumental spines, scale bar = 100 µm.

Table III.1

Metric data for Intromugil alachuaensis n. sp. and Intromugil mugilicolus from new material and for Intromugil mugilicolus, Intromugil simonei, and Intromugil annakohnae from published reports. Min-Max, Mean (N).

Measurement	*I. alachua-ensis*n. sp.*	*I. mugilicolus**	*I. mugilicolus* Shireman (1964)	*I. simonei* Travassos, Frietas, & Bührnheim (1965)	*I. annakohnae* Ferandes & Cohen (2006)	*I. annakohnae* Conroy & Conroy, (1986)
Length (BL)	1772-3181 2420 (6)	1371-2106 1771 (10)	2542-2772	2111-2560	700-1900 1260 (16)	833-1613
Body width	376-533 465 (5)	270-553 468 (6)	715-744	800-910	300-620 690 (16)	271-499
Body depth	488 (1)	312-429 364 (4)				
Forebody	652-783 728 (6)	427-650 516 (10)	570-885			
Hindbody	893-1578 1323 (6)	796-1369 1104 (10)	1380-1710			
Precaecal bifurcation distance	754-1151 1007 (6)	669-926 802 (10)				
Postcaecal distance	220-447 327 (6)	300-464 368 (10)		560-850		
Oral sucker (OS) length	278-312 301 (6)	105-159 131 (10)	140-175	250	75-175 158 (16)	94-147
OS width	257-276 266 (5)	137-201 178 (6)	200-220	320-330	100-230 178 (16)	106-159
OS depth	200 (1)	105-124 114 (4)				
Ventral sucker (VS) length	164-245 213 (6)	104-175 147 (10)	165-220	200-210	87-175 128 (12)	74-133
VS width	148-256 266 (5)	121-199 175 (6)	198-253	200-210	97-175 133 (12)	82-140
VS depth	207 (1)	103-120 114 (4)				
Prepharynx length	136-290 241 (6)	181-356 271 (10)	300-355	Very short	10-70 35 (14)	55
Pharynx length	235-272 254 (6)	126-219 181 (10)	205-230	150-170	50-115 81 (16)	71-116
Pharynx width	144-196 172 (6)	110-173 144 (10)	170-220	160-170	55-130 85 (16)	72-106
Esophagus length	116-253 195 (6)	159-259 224 (10)	198-253	590-670	140-450 316 (13)	136-292
Testis length	366-565 493 (6)	248-575 394 (10)	455-630	380-520	105-410 251 (13)	154-278
Testis width	161-312 258 (6)	151-371 253 (10)	220-300	320-547	115-230 162 (13)	101-199
Testis to VS	293-707 587 (6)	329-559 450 (10)				
Post-testicular space	215-341 259 (6)	163-341 278 (10)	150-345			

Table III.1 (continued).

Measurement	*I. alachuaensis* n. sp.*	*I. mugilicolus**	*I. mugilicolus*	*I. simonei*	*I. annakohnae*	*I. annakohnae*
Ovary length	123-158	62-155	155-160	130-170	42-215	
	142 (5)	108 (10)			111 (10)	
Ovary width	88-116	67-178	95-120	150-160	30-110	41-83
	105 (5)	114 (10)			70 (10)	
Ovary to VS	350-450	221-438				
	399 (5)	347 (10)				
Vitellarium to	72-440	117-337				
VS	316 (6)	190 (10)				
Vitellarium to	39-80	25-103				
posterior end	64 (6)	61 (10)				
Hermaphroditic	280-477	233-473	270-335	330	140-450	114-176
sac length	389 (6)	388 (10)			238 (14)	
Hermaphroditic	125-157	109-192	60-80	150-180	60-145	
sac width	149 (6)	162 (10)			107 (14)	
Internal seminal	170-301	122-283			60-340	
vesicle length	234 (6)	213 (10)			184 (9)	
Internal seminal	73-135	42-172			30-150	
vesicle width	109 (6)	113 (10)			69 (9)	
External	146-248	124-228		170	60-215	
seminal vesicle	180 (5)	186 (10)			110 (13)	
length						
External	82-152	59-176			30-115	
seminal vesicle	114 (5)	113 (10)			61 (13)	
width						
Egg length	71-86	67-73	60-80	90-100	82-105,110	91-116
	76 (19)	70 (10)			93 (53)	
Egg width	38-48	38-47	40	53-57	45-72, 67	57
	43 (19)	42 (10)			54 (53)	
Width †	17-21%	18-28%				
	19 % (5)	25% (6)				
Forebody†	24-37%	25-34%				
	31% (6)	29% (10)				
Hindbody†	40-65%	58-65%				
	55% (6)	62% (10)				
Precaecal	33-49%	40-50%				
bifurcation†	42% (6)	46% (10)				
Postcaecal	9-20%	17-25%				
distance†	14% (6)	21% (10)				
OS length %	113-190%	74-101%				
VS length	144% (6)	90% (10)				
OS width % VS	107-175%	93-113%			60-80%	
width	130% (5)	102% (6)			70% (12)	
Pharynx length/	86-193%	46-104%				
prepharynx	113% (6)	69% (10)				
length						
Pharynx length	124-183%	111-139%				
/ Pharynx width	150% (6)	126% (10)				
Prepharynx/	91-189%	97-156%				
esophagus	127% (6)	122% (10)				

Table III.1 (continued).

Measurement	*I. alachuaensis* n. sp.*	*I. mugilicolus**	*I. mugilicolus*	*I. simonei*	*I. annakohnae*	*I. annakohnae*
Testis to VS distance†	17-29% 24% (6)	22-32% 26% (10)				
Post-testicular space†	7-14% 11% (6)	10-19% 16% (10)				10-14%

* Non-flattened specimens from this study

†as percentage of body-length

Table III.2.

Total number of variable sites based on pairwise comparision of the studied fragment of nuclear rDNA (partial 18S, complete ITS1, complete 5.8S, complete ITS2 and partial 28S) between Intromugil mugilicolus and Intromugil alachuaensis n. sp.

	Intromugil alachuaensis length	*Intromugil mugilicolus* length	Aligned length	Aligned base pair differences	Gaps	Percent difference
18S partial	133	133	133	1	0	0.8%
ITS1	504	506	508	42	6	9.4%
5.8S	157	157	157	0	0	0
ITS2	287	287	287	17	0	5.9%
28S partial	1381	1382	1384	39	5	3.2%
Total	2462	2465	2469	99	11	4.5%

the genus, (2) the considerably longer prepharynx is than in *I.annakohnae* and *I. simonei*, (3) the robust and elongate compared with pyriform pharynx in *I. mugilicolus*, or smaller pharynx with a nearly equal length to width ratio as in *I. annakohnae* and *I. simonei*, (4) the less extensive vitellarium than in *I. mugilicolus*, and (5) the caeca that terminate at the level of the posterior half of the testis rather than at or near the anterior testicular margin like in *I. mugilicolus*, or anterior to the testis in *I. annakohnae* and *I. simonei*.

Intromugil alachuaensis, possibly sympatric with *I. mugilicolus*, is much less transparent than that species both in life and in mounts. The eye-spot pigment in *I. alachuaensis* is much denser throughout the body, and it has many glands in the forebody

that significantly darken the appearance. Both *I. alachuaensis* and *I. mugilicolus* have two large collecting lymphatic ducts extending roughly parallel to the prepharynx, pharynx, and oesophagus, but the tubules in *I. alachuaensis* are larger and more prominent in both live and fixed material than in *I. mugilicolus*. *Intromugil alachuaensis* appears more robust than the other three members of the genus.

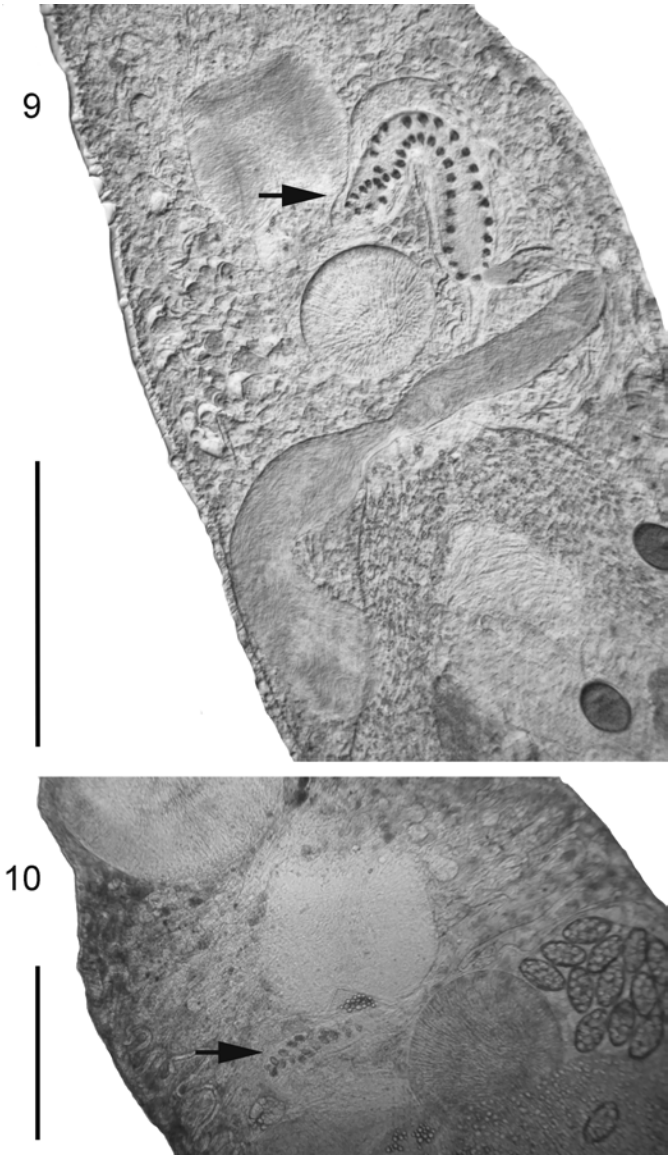
Molecular Data

According to the available information on the sequenced contiguous fragment, these regions and especially the highly variable ITS1 are generally suitable for the discrimination of congeneric digenean species (e.g. Nolan & Cribb, 2005; Olson & Tkach, 2005; Parker et al., 2010; Tkach & Kinsella; 2011; Tkach & Mills, 2011). No intraspecific variability was detected among replicates of either species. The region sequenced and used for differentiation (Table 2) when aligned and trimmed was 2,477 bp for *I. mugilicolus* and 2,474 bp for *I. alachuaensis,* and it differed at 110 sites across all sequenced regions, breaking down as follows: 18S 133 bases, 1 difference; ITS1, 48 differences, including 6 gaps; 5.8S 0 difference; ITS2, 17 differences; and 28S 44 differences, including 5 gaps.

Discussion

Prior to this study, *Intromugil* contained 3 nominal species from fishes in the genus *Mugil*, all found in water bodies associated with the Western Atlantic Ocean in both North and South America. Overstreet and Curran (2005) considered *Skrjabinolecithum* Belous, 1954 to be the eastern hemisphere counterpart of *Intromugil*, and differentiated them by geography, the extent of the caeca, and position of the ovary

relative to testis. With the addition of *I. alachuaensis* and new material of *I. mugilicolus*, geography and caecal extent still serve to differentiate the two genera. In material of *I.*



Figures III.9-10. Nomarski photomicrographs of living *Intromugil* material. 9. *Intromugil mugilicolus* showing glands in hermaphroditic duct, scale bar = 200 µm. 10. *Intromugil alachuaensis* n. sp. showing glands in hermaphroditic duct, arrows indicate sacs containing glandular material, scale bar = 200 µm.

mugilicolus that has not been flattened, the ovary is not distinctly pretesticular, but rather contiguous with the testis or only slightly pretesticular. In *I. alachuaensis*, the ovary is up

to about one ovarian length pretesticular. In the current study, I observed the excretory vesicle of *I. mugilicolus* and *I. alachuaensis* as I-shaped, which was previously unreported. Species in *Skrjabinolecithum* have a Y-shaped excretory vesicle. Of sympatric species in haploporid genera in mullet, those of *Culuwiya* Overstreet & Curran, 2005 are more commonly found, but they are smaller, possess shorter sac-like caeca, and have a Y- shaped excretory vesicle. Overstreet and Curran (2005) placed *Intromugil* into the subfamily Waretrematinae, whose members are largely confined to the western Pacific and Indian Ocean Basin, with the exception of species in the genera *Intromugil*, *Culuwiya* and *Conohelmins* from the Americas. I suspect that once molecular data become available for waretematines from the eastern hemisphere, *Culuwiya* and *Intromugil* will be more closely allied with the chalcinotrematines than with other waretrematines. As species of haploporids are found predominantly in mullets throughout their range, I expect to detect considerable convergence of morphological features in different geographical areas from different lineages.

Unfortunately, I do not have fresh, properly-killed material from *I. simonei* and *I. annakohnae*. Specimens of *I. mugilcolus* killed with pressure are inconclusive in demonstrating three rows of spines on a terminal oral sucker, whereas the flattened large muscular oral sucker of *I. alachuaensis* retains its shape and position. Additional material from *I. simonei* and *I. annakohnae* is needed to determine if three rows of spines occur on the oral sucker and if the sacs of glandular material occurring in the hermaphroditic duct are specific or generic characteristics. I suspect both features to be present in *I. simonei* and *I. annakohnae*. Conroy and Conroy (1986) reported *I. mugilicolus* from *M. curema* in Venezuela. Based on their description of a short prepharynx and small pharynx in worms

not available for examination, I think their worms were *I. annakohnae*. They reported pronounced muscularization visible in the hermaphroditic duct and that alludes to the sacs I observed in *I. mugilicolus* and *I. alachuaensis*. As I already mentioned, these are best observed in living worms. Molecular data also are needed to determine the pattern of diversification of *Intromugil* spp. Molecular data from the two South American species would help delineate how diversification in *Intromugil* species evolved and whether the group has a Neotropical or Nearctic origin.

Some morphological features of the new species and redescription of *I. mugilcolus* with unflattened specimens warrants emendation of the generic diagnosis of the genus provided by Overstreet and Curran (2005) to include oral sucker as terminal to subterminal (vs. terminal in current diagnosis), oral sucker with or without three rows of small spines along anterior margin of mouth within oral sucker, caeca terminating at a level from anterior to testis to posterior half of testis (vs. anterior margin of testis), and ovary as contiguous with to anterior to testis (vs. anterior to testis).

Intromugil mugilcolus collected in Mississippi appears to be rare because many fish have been examined in recent years. *Mugil cephalus* seems to be infected only in winter and spring, from roughly January to April. The parasites appear to occur only in fish larger than about 250 mm in total length (TL) with relatively low intensity. For many years before Hurricane Katrina (August 2005) and until March 2007 it has not been found in any of numerous mullet examined (Overstreet, 2007). I do not present prevalence data because of the temporal absence of this parasite and its seeming restriction to large mullets. During the time of year when I find *I. mugilicolus*, the salinity tends to be lower than during the rest of the year, but the period also immediately follows that of the

offshore spawning migration of *M. cephalus*. In my sample of *M. cephalus* that yielded specimens of *I. alachuaensis* from the Santa Fe River, four fish measured 405-550 mm TL and were infected with one to six specimens. Mullet smaller than about 300 mm were not observed during the time of collection. The four mullet that harbored *I. alachuaensis* were captured more than 80 km inland. Although infections of *I. alachuaensis* could have been acquired in marine waters, I suspect that they were acquired in freshwater. *Intromugil alachuaensis* is a large and distinct worm, and previous investigations of Florida marine waters (Hutton & Sogandares-Bernal, 1964; Hutton, 1964; Skinner, 1975) failed to uncover this species or *I. mugilicolus*. No larval form of any species of *Intromugil* has been reported. Further investigation is needed into the life-cycle and whether *I. alachuaensis* occurs in other rivers with similar geology in peninsular Florida.

CHAPTER IV

SOME NEW AND OTHER SPECIES OF HAPLOPORIDAE (DIGENEA) POSSESSING A SINGLE CAECUM INFECTING MUGILID FISHES, WITH PHYLOGENETIC AFFINITIES

Abstract

Nine species of Haploporidae, possessing an undivided caecum from hosts in Mugilidae are reported, four of which are new. In this study, it is concluded that the Indo-Pacific haploporids with a caecum are closely related, requiring several changes to the prior organization of the Haploporidae. *Malabarotrema* Zhukov, 1972 is resurrected for *Malabarotrema indica* Zhukov, 1972; *Skrjabinolecithum lobolecithum* Martin, 1973 is transferred to *Malabarotrema* as M. *lobolecithum* (Martin, 1973) n. comb.; *Pseudohapladena megaorchis* Liu and Yang, 2002 is transferred to *Malabarotrema* as *M. megaorchis* (Martin, 1973) n. comb.; and a new species is described. Species of *Unisaccus* Martin, 1973 are transferred out of the Haploporinae to the Waretrematinae on the basis of morphological and molecular data. *Unisaccoides* Martin, 1973 is resurrected with *Unisaccoides vitellosus* Martin, 1973 as the type-species, and a new species of *Unisaccoides* is described. *Saccocoelioides lizae* Liu, 2002 is transferred to *Unisaccus* as *Unisaccus lizae* (Liu, 2002) n. comb., and three new species are described. Phylogenetic hypotheses based on analysis of partial 28S sequences with other available haploporid sequences indicate that (1) members of *Malabarotrema, Unisaccoides*, and *Unisaccus* are more closely related to each other than to other species of haploporids; (2) Haploporinae is not a monophyletic group when species of *Unisaccus* are included; (3) *Saccocoelioides lizae* is better allocated to *Unisaccus* than to *Saccocoelioides*; and (4) *Skrjabinolecithum*

vittellosus and five species of *Unisaccus* form a monophyletic group. Reduced vitellarium and the presence of eye-spotted miracidium in-utero are no longer considered characters that exclude species from Waretrematinae.

Introduction

The subfamily Unisaccinae Martin, 1973 was originally erected for species that possess an undivided caecum, accommodating the genera *Unisaccus* Martin, 1973 and *Unisaccoides* Martin, 1973 (Martin, 1973b). Martin also described *Paraunisaccoides* Martin, 1973, which he also placed in the subfamily (Martin, 1973c). Martin (1973b, c) apparently was unaware of the description of *Malabarotrema indica* Zhukov, 1972 by Zhukov (1972). *Malabarotrema* shares many features with *Paraunisaccoides*. The genus *Pseudouncoelium* Ahmad, 1987 was erected for two species described from mugilids off India that also possessed a caecum and were also included in the subfamily (Ahmad, 1987). Overstreet and Curran (2005) considered *Pseudounicoelium* Ahmad, 1987 a junior synonym of *Unisaccus* and did not accept the subfamily; they considered it a junior synonym of Haploporinae Nicoll, 1914. Overstreet and Curran (2005) considered *Malabarotrema*, *Unisaccoides,* and *Paraunisaccoides* as junior synonyms of *Skrjabinolecithum* Belous, 1954, a member of Waretrematinae Srivastava, 1937. Although they noted the similarity of *Pseudohapladena megaorchis* Liu & Yang, 2002 to the concept of *Paraunisaccoides,* they retained *Ps. megaorchis* in *Pseudohapladena* Yamaguti, 1952. Blasco-Costa, Gibson et al. (2009) and Blasco-Costa, Montero, Gibson, Balbuena, and Kostadinova (2009) retained *Unisaccus* within the Haploporinae and transferred two additional species into the genus as *Unisaccus sprenti* (Martin, 1973) Blasco-Costa, Mantero, Gibson, Balbuena, Raga, and Kostadinova, 2009 and *Unisaccus*

mugilis (Rekharani & Madhavi, 1985) Blasco-Costa, Mantero, Gibson, Balbuena, Raga, and Kostadinova, 2009. *Unicoelium* Thatcher and Dossman, 1975 was placed into the subfamily Chalcinotrematinae Overstreet and Curran, 2005 by Overstreet and Curran (2005), even though Nasir and Gómez (1976) had previously considered *Unicoelium* as a synonym of *Unisaccus*. Overstreet and Curran (2005) considered the single caecum to have evolved independently in at least three of the four haploporid subfamilies they recognized.

In this paper, I report on nine species of haploporids from the Indo-West Pacific region and attempt to place them within a larger framework of the Haploporidae Nicoll, 1914, as well as provide keys to species with an undivided caecum in mullets.

Materials and Methods

Specimens of Haploporidae with a caecum were collected from mugilid (Actinopterygii: Mugiliformes) hosts collected at several locations off Australia and purchased from fish markets in China (assumed to be wild caught). Fish names follow those of FishBase (Froese & Pauly, 2013).

Recently collected haploporids (since 2009) were isolated similarly to the process advocated by Cribb and Bray (2010) for gastrointestinal species, often skipping an initial examination because of the volume of the intestinal contents. Live worms were rinsed and cleaned in saline and observed briefly. Worms were killed by addition of steaming hot, but not boiling, tap water. Haploporids were then stored in 70% molecular-grade ethanol. Parasite specimens for morphological and molecular analysis were processed and studied according to the protocols used by Pulis et al. (2013) and Pulis and Overstreet (2013). Prepared permanent slides also were obtained from the personal collections of

Thomas Cribb and Robin Overstreet. All measurements are in micrometres unless noted otherwise. Museum abbreviations are as follows: MNT, Museum and Art Gallery of the Northern Territory, Darwin, Australia; QM, Queensland Museum, Brisbane, Queensland, Australia; and USNPC, US National Parasite Collection, Beltsville, Maryland.

Genomic DNA was isolated using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, U.S.A.) following the instructions provided. DNA fragments approximately 2,500 basepairs (bp) long comprising the 3' end of the 18S nuclear rDNA gene, internal transcribed spacer region (including ITS1 + 5.8S + ITS2), and the 5' end of the 28S 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 primers ITSF (5' - CGCCCGTCGCTACTACCGATTG-3') or LSU5 (5'-TAGGTCGACCCGCTGAAYTTAAGCA-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'), 900F (5'-CCGTCTTGAAACACGGACCAAG-3'), and internal reverse primers were 300R (5'-CAACTTTCCCTCACGGTACTTG-3'), Digl2r (5'-CCGCTTAGTGATATGCTT-3'), and ECD2 (5'-CTTGGTCCGTGTTTCAAGACGGG-3').

Previously published 28S ribosomal RNA gene sequences of species of Haploporidae were used for comparison (see Table 1 for accession numbers and host information) with newly submitted sequences. Sequences were aligned using the ClustalW application in the BioEdit program, Version 7.0.9 (Hall, 1999). The alignment

was further refined by eye and trimmed to the shortest sequence on both 5' and 3' ends. The resulting alignment utilised 29 haploporids with *Hapladena nasonis* as the outgroup. The alignment was 1,183 characters long, including gaps, with 743 sites conserved, 435 sites variable, and 325 parsimony-informative sites. Phylogenetic analysis of the data was performed using Bayesian inference (BI) with MrBayes 3.1.2 software (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The best nucleotide substitution model was estimated with jModeltest Version 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) as general time reversible, with 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*. The first one quarter of samples were discarded as burn-in (*sump burnin = 2500*), and nodal support was estimated by posterior probabilities (*sumt*) (Huelsenbeck et al., 2001), with all other settings left as default.

Results

Malabarotrema Zhukov, 1972

Syn Paraunisaccoides Martin, 1973

Diagnosis: Body elongate. Eye-spot pigment dispersed in forebody. Oral sucker subterminal. Prepharynx longer than pharynx and oesophagus. Oesophagus short. Caecum single, longer than wide, extending to near or into testicular region, with posterior indentation. Testis in hindbody. Hermaphroditic sac dorsal to ventral sucker, often widest in anterior half. Hermaphroditic duct with distal portion composed of smooth muscle, proximally lined with muscular pads. Female duct short. Internal seminal vesicle about half length of hermaphroditic sac. External seminal vesicle usually longer

than internal seminal vesicle. Ovary contiguous with or short distance anterior to testis. Uterus confined between ovary and hermaphroditic sac. Vitellarium composed of large areas of elongate follicles, confined mainly to hindbody. In-utero eggs with incompletely developed miracidium lacking eye-spots. Excretory vesicle Y-shaped, bifurcating in testicular region; arms reaching to middle of hindbody; pore terminal. In intestine of Mugilidae from Indo-West Pacific region.

Type-species: *Malabarotrema indica* Zhukov, 1972

Remarks

The type-species of *Malabarotrema* had previously been allocated to *Skrjabinolecithum* by Overstreet and Curran (2005).

Malabarotrema indica Zhukov, 1971

Syns Skrjabinolecithum indicum (Zhukov, 1971) Overstreet and Curran, 2005.

Type-host: Etroplus suratensis (Bloch), pearlspot (Cichlidae).

Type-locality: Malabar, India.

Site of infection: Intestine.

Specimens examined: Russian Academy of Sciences, No. 46-4, 2 specimens on one slide. ex. *Etroplus suratensis* (Cichlidae) Malabar, India.

Examination of slide 46-4 RAS reveals that *Malabarotrema indica* has a caecum. Zhukov's (1972) Figure 3 is accurate, whereas Overstreet and Curran (2005) misinterpreted the wide oesophagus as the bifurcation of the oesophagus prior to the sac-like caecum. Examination of the specimens also revealed the presence of pads lining the hermaphroditic duct as in the other species of *Malabarotrema*. I consider those two

features along with the extensive vitellarium to necessitate the resurrection of *Malabarotrema* from *Skrjabinolecithum.*

Because the type specimens of *M. indica* were killed under considerable coverslip pressure, I infer the position and nature of the organs from the specimens I collected from the other three species I place in the genus.

Malabarotrema lobolecithum (Martin, 1973) n. comb.

Syns Paraunisaccoides lobolecithum Martin, 1973; *Skrjabinolecithum lobolecithum* (Martin, 1973) Overstreet & Curran, 2005

Type-host: *Mugil cephalus* Linnaeus, flathead grey mullet (Mugilidae).

Type-locality: Brisbane River, Brisbane, Queensland, Australia. (Martin, 1973c)

Site of infection: intestine.

Other hosts: *Paramugil georgii* (Smith), silver mullet (Mugilidae); *Moolgarda seheli* (Forsskål), bluespot mullet (Mugilidae); *Moolgarda perussii* (Valenciennes), longfinned mullet (Mugilidae).

Material examined: *ex. Paramugil georgii*; Western Australia, Australia; Barred Creek (17° 40'S 122° 12'E) February 2010, Coll. Pulis, Andres, Six Mile Creek 20° 19'S 118° 40'E Feburary2010 Coll. Pulis, Andres, Northern Territory, Australia; Ludmilla Creek (12° 25'S 130° 50'E) March 2010, Coll. Pulis, Andres. Queensland, Australia; Woody Point (27° 16'S 153° 06'E) Coll. Overstreet February 1995, Sandgate (27° 19'S 153° 05'E) Coll. Clarke, Cribb, Maguire January 1995, Boat Passage (27° 24'S 153° 10'E) Coll. Cribb, Anderson, Bray January 1993, Wynnum (27° 26'S 153° 11'E) Coll. Pulis, Andres March 2010, Wynnum (27° 27'S 153° 10'E) Coll. Cribb et al. February 1995, Wynnum Purchased fresh Overstreet Nov. 1997, Logan River (27° 42'S 153° 19'E) Coll.

Overstreet February 1995. *ex. Moolgarda seheli*: Western Australia, Australia; Dampier Creek (17° 57'S 122° 15'E) February 2010 Coll. Pulis, Andres, Queensland, Australia; Beelbi Creek (25° 15'S 152° 40'E) Coll. Pulis, Andres March 2010. *ex. Moolgarda perussii*: Northern Territory, Australia, Doyle's Boat Ramp (12° 26'S 130° 50'E) Coll. Pulis, Andres March 2010.

Specimens deposited: USNPC NTM BM-TBD, representative sequence, EP-568.

Holotype: No. 7114 Allen Hancock Parasitology Collection, University of Southern California, but current whereabouts unknown; unsuccessful attempts were made to borrow specimens from University of Southern California Museum, Santa Barbara Museum of Natural History, and Los Angeles County Museum of Natural History (Pers. comm. Daniel Geiger SBMNH, Jody Martin NHMLAC).

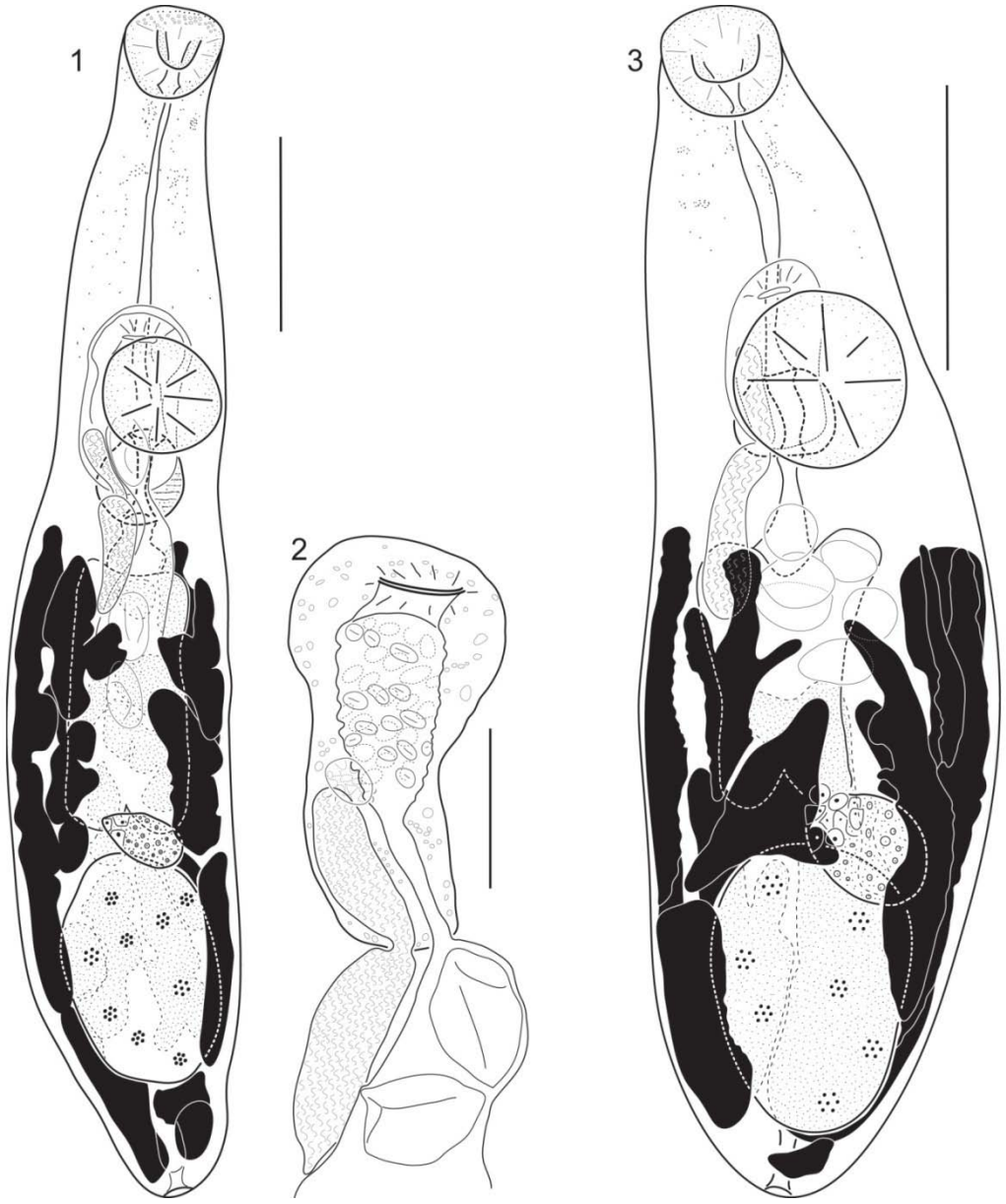
Supplementary Data (Figures 1-2, Tables 1-3).

Measurements based on 11 wholemount specimens. Body elongate, fusiform, 1,270-2,005 long, 219-366 wide, widest in hindbody, width 14-21% of body length (BL), whitish in life. Tegument spined, with ventral surface between ventral sucker and oral sucker containing only few irregularly spaced spines; spines bent-hastate, about 9-13 long, 6-8 wide at widest point of root, directed posteriorly. Dorsal to oral sucker 3 rows of enlarged spines, blunt, conical, with bases about 7-11 wide. Eye-spot pigment dispersed in forebody, most prominent in anterior fifth of body. Oral sucker subterminal, 99-140 long, 92-163 wide, with mouth opening anteroventrally. Ventral sucker slightly elevated, 115-181 long, 136-204 wide, 113-147% longer than oral sucker, 106-157% wider than oral sucker, covered by spines; spines covering ventral sucker spines smaller than body spines, similar in shape. Forebody 351-549 long or 25-31% of BL; hindbody

774-1284 long or 60-67% of BL. Prepharynx well defined, 327-685 long. Pharynx thick-walled, muscular, usually pyriform, located dorsal to completely posterior to ventral sucker, 91-144 long, 90-150 wide; prepharynx 3.3-5.2 times longer than pharynx, with posterior portion and pharynx surrounded by few gland cells probably of digestive nature. Oesophagus not as well defined as prepharynx, often folded slightly among other organs, 43-161 long. Caecum in hindbody, lined with large cells, with anterior end expanded, with posterior end slightly indented, 268-498 long, 132-210 wide, commencing 567-1,001 from anterior end of body or 41-53% of BL; post-caecal space 235-619 or 18-36% of BL.

Testis medial, with anterior margin straight or beveled, with posterior end rounded to pointed, overlapping posterior end of caecum to 139 posterior, 257-644 from ventral sucker, 230-397 long, 136-207 wide; post-testicular space 98-395 or 6-20% of BL. Hermaphroditic sac dorsal to ventral sucker, ventral to pharynx, 190-315 long, 87-145 wide, terminating posterior to ventral sucker; sac containing following structures (internal seminal vesicle 42-148 long, 30-60 wide; male duct thin-walled; prostatic bulb prominent, with size dependent on amount of sperm and eggs present; male duct joining female duct in posterior half of sac); hermaphroditic duct strongly muscularised, with posterior portion containing muscular pads. External seminal vesicle sac-like, variously orientated, 86-152 long, 38-83 wide. Genital pore elongate laterally, without evidence of scleritization, located at about level of anterior margin of ventral sucker in crease between elevated ventral sucker and of plane forebody.

Ovary medial, contiguous with testis, 94-148 long, 61-104 wide, 257-644 from ventral sucker. Mehlis' gland slightly anterior to ovary. Laurer's canal dorsal to testis,



Figures IV.1-3 Malabarotrema lobolecithum and *Malabarotrema megaorchis* 1. *Malabarotrema lobolecithum* ventral wholemount; 2. *Malabarotrema. lobolecithum* hermaphroditic sac of specimen killed while under coverslip pressure; 3. *Malabarotrema megaorchis* ventral wholemount. *Scale-bars* 1, 250µm; 2, 200µm; 3, 250µm.

ventral to caecum; pore dorsal, near mid-testicular level. Vitellarium consisting of numerous large follicles, usually commencing posterior to margin of ventral sucker (1 exceptional specimen at margin of ventral sucker) to 275 posterior to ventral sucker (averaging 106), evenly distributed dorsally and ventrally, not confluent ventral to testis

or uterus, confluent posterior to testis. Uterus extending directly from ovary to hermaphroditic sac, without loops; proximal portion often filled with sperm; metraterm lacking. Eggs thin-shelled, 1-17 in number, 75-106 long, 40-67 wide (averaging 93 X 55) when measured from distal end of uterus, with incompletely developed miracidium lacking eye-spots.

Lymphatic tubes in forebody. Excretory vesicle Y-shaped, dorsal to testis, bifurcating near mid-level of testis, terminating near anterior margin of ovary; pore terminal.

Remarks

Martin (1973c) described the species from two specimens. I included additional specimens in my series, accounting for greater variability in measurements and metrics. There was no major difference from the original description other that I could not confidently observe the sclerotized denticles on/in the pads lining the posterior portion of the hermaphroditic duct. I believe them to be glands or gland products of an uncertain function and not apparent in specimens fixed by both methods. Although the species was originally described from *Mugil cephalus,* I did not find this species in *M. cephalus* but have encountered it in other mullet species. I believe the host was either originally misidentified or from an irregular host; regardless, I have no reason to think my worms are not conspecific with those described by Martin (1973c).

Malabarotrema megaorchis (Liu & Yang, 2002) n. comb.

Syn Pseudohapladena megaorchis Liu and Yang, 2002.

Type-host: *Moolgarda engeli* (Bleeker), Kanda (Mugilidae); reported as *Mugil engeli* by Liu and Yang (2002).

Type-locality: Xiamem, Fuijian Province, People's Republic of China.

Site of infection: Intestine.

Material Studied: Paratype, USNPC No. 91532.

New Material: ex. *Mugil cephalus* purchased at fish market, Daya Bay (22° 43'N 114°32'E), Guangdong Province, People's Republic of China.

Specimens deposited: TBD, represenative sequence, EP-644.

Supplementary Data (Figures, 3,20; Tables, 1-3)

Measurements based on 5 wholemount specimens. Body elongate, fusiform 951-1075 long, 257-336 wide, widest in hindbody, with width 27-32% of BL. Tegument spined over entire surface; spines becoming smaller and more sparse posteriorly, hastate, about 4-8 long (measured from forebody), directed posteriorly. Eye-spot pigment dispersed in forebody, most prominent in anterior half of forebody. Oral sucker subterminal, 91-100 long, 98-115 wide, with mouth opening anteroventrally. Ventral sucker slightly elevated, 130-155 long, 130-159 wide, 135-170% longer than oral sucker; 125-159% wider than oral sucker, covered by spines; spines covering ventral sucker smaller than body spines, similar in shape. Forebody 251-343 long or 25-32% of BL, hindbody 519-623 long or 53-62% of BL. Prepharynx well defined, 211-294 long. Pharynx thick-walled, muscular, usually pyriform, located dorsal to ventral sucker to partially dorsal posterior to posterior margin of ventral sucker, 79-98 long, 92-109 wide; prepharynx 2.5-3.2 times longer than pharynx, with posterior portion of prepharynx and pharynx surrounded by few gland cells of probable digestive nature. Oesophagus not as well defined as prepharynx, often folded slightly among other organs, 32-98 long. Caecum in hindbody, lined with large cells, (specimen in Figure 3 anterior end expanded

and protruding anteriorly due to displacement by eggs in uterus), with posterior end indented, 223-272 long, 111-145 wide, commencing 461-521 from anterior end of body or 46-50% of BL; post-caecal space 273-308 or 26-30% of BL.

Testis medial, rounded, overlapping posterior end of caecum to 25 distant, 228-336 from ventral sucker, 205-240 long, 132-170 wide; post-testicular space 54-138 or 5-13% of BL. Hermaphroditic sac dorsal to ventral sucker, ventral to pharynx, 161-186 long, 72-89 wide, terminating near posterior margin ventral sucker; sac containing following structures (internal seminal vesicle 60-89 long, 28-36 wide; male duct thin-walled; prostatic bulb prominent, with size dependent on amount of sperm and eggs present; male duct joining female duct in posterior half of sac); hermaphroditic duct strongly muscularized, with posterior portion containing muscular pads. External seminal vesicle sac-like, 98-162 long, 28-43 wide. Genital pore immediately anterior to ventral sucker in crease between elevated ventral sucker and plane of forebody.

Ovary medial, ventral to caecum, situated laterally along ventral anterior margin of testis, 94-121 long, 60-84 wide, 166-288 from ventral sucker. Mehlis' gland anterior or lateral to ovary. Laurer's canal not observed. Vitellarium plate-like or tubular, usually commencing at posterior margin of ventral sucker (1 specimen at 31 posterior to posterior margin of ventral sucker), confluent posterior to testis. Uterus extending directly from ovary to hermaphroditic sac, without loops; proximal portion often filled with sperm; metraterm lacking. Eggs thin-shelled, 2-16 in number, 58-71 long, 42-51 wide (average 64 X 46) when measured from distal end of uterus, with incompletely developed miracidium lacking eye-spots.

Lymphatic tubes in forebody. Excretory vesicle Y-shaped, dorsal to testis, bifurcating near mid-level of testis, terminating near anterior margin of ovary; pore terminal.

Remarks

The smaller, stouter, *Malabarotrema megaorchis* can be differentiated from *M. lobolecithum* by geography, smaller body spines, a lack of enlarged spines dorsal to oral sucker, having smaller eggs, and molecular differences.

Malabarotrema n. sp. 1

Type-host: *Paramugil georgii* (Ogilby), silver mullet (Mugilidae).

Type-Locality: Queensland, Australia: Beelbi Creek (25° 15'S 152° 40'E).

Site: Intestine.

Other hosts: Moolgarda seheli (Forsskål), bluespot mullet (Mugilidae); *Paramugil georgii* (Ogilby), silver mullet (Mugilidae).

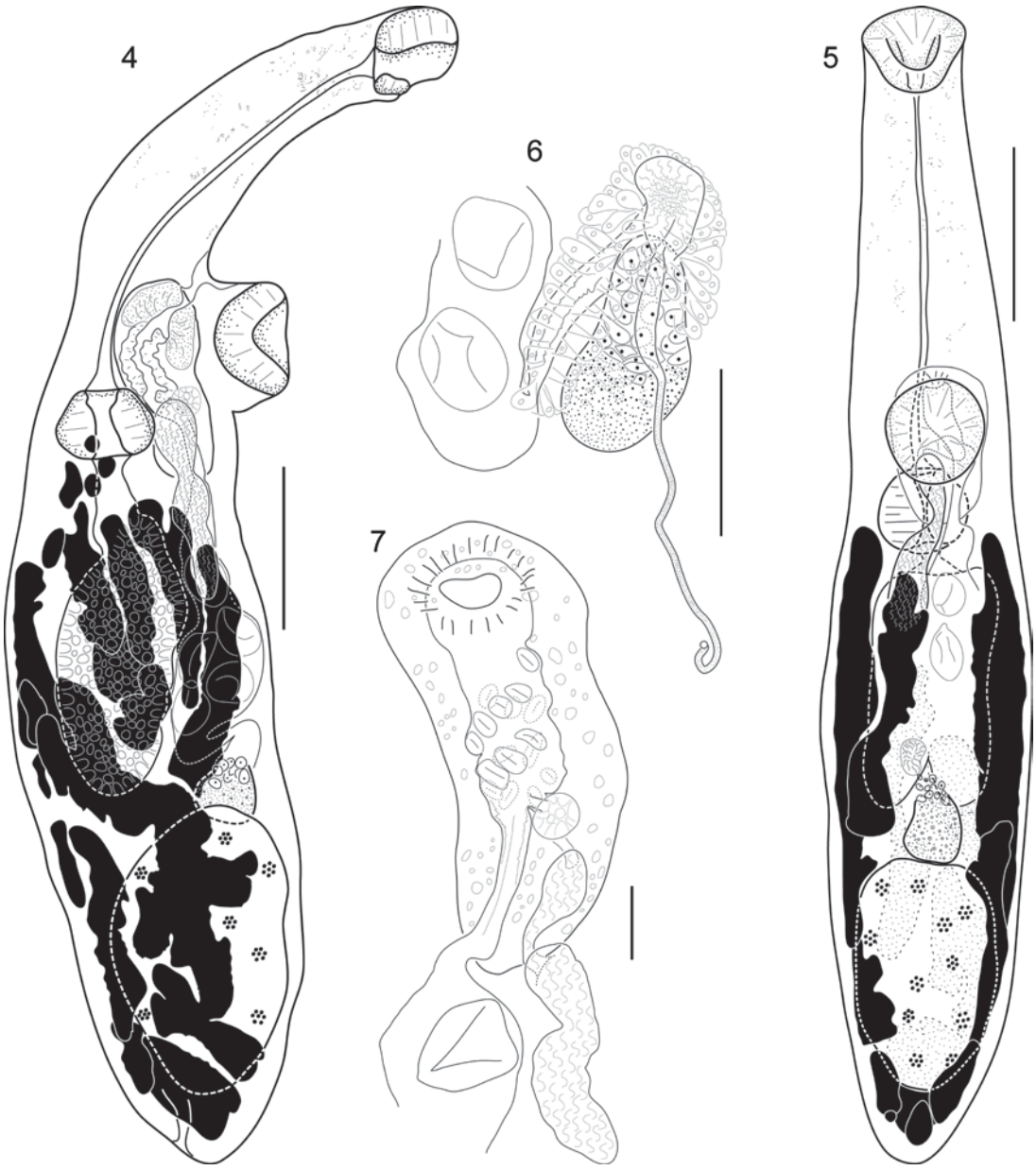
Other localities: ex. Paramugil georgii; Northern Territory, Australia: Ludmilla Creek (12° 25'S 130° 50'E) Coll. Pulis, Andres March 2010, Queensland, Australia: Beelbi Creek (25° 15'S 152° 40'E) Coll. Pulis, Andres March 2010, Eli Creek (25° 16'S 152° 49'E) Coll. Pulis, Andres March 2010, Woody Point (27° 16'S 153° 06'E) Coll. Overstreet February 1995, Sandgate (27° 19'S 153° 05'E) Coll. Clarke, Cribb, Maguire January 1995, Morton Bay (27° 21'S 153° 07'E) Coll. Cribb, Barker, Anderson, Wright July 1999, Boat Passage (27° 24'S 153° 10'E) Coll. Cribb, Anderson, Bray January 1993, Wynnum (27° 26'S 153° 11'E) Coll. Pulis, Andres March 2010, Wynnum (27° 27'S 153° 10'E) Coll. Cribb et al. February 1995, Logan River (27° 42'S 153° 19'E) Coll. Overstreet February 1995. *ex. Moolgarda seheli*: Northern Territory, Australia: Buffalo Creek (12°

12'S 130° 55'E) Coll. Pulis, Andres March 2010, Queensland, Australia: Eli Creek (25° 16'S 152° 49'E) Coll. Pulis, Andres March 2010, Wynnum (27° 26'S 153° 11'E) Coll. Pulis, Andres March 2010.

Specimens Deposited: Holotype: TBD Paratypes USNPC NTM BM TBD, representative sequence, EP-148.

Description Figures 4-7, Tables 1-3.

Measurements based on 15 wholemount specimens. Body elongate, fusiform 897-1,644 long (BL), 177-370 wide, widest in hindbody, width 18-27% of BL. Tegument spined over entire surface, except for area surrounding genital pore; spines hastate, about 6-9 long, 2-4 wide at widest point of root, directed posteriorly. Eye-spot pigment dispersed in forebody, most prominent in anterior fifth of body. Oral sucker subterminal, 70-120 long, 91-149 wide, with mouth opening anteroventrally. Ventral sucker slightly elevated, 98-157 long, 107-161 wide, 115-154% longer than oral sucker, 93-148% wider than oral sucker, covered by concentric rows of spines; spines covering ventral sucker similar to body spines, smaller than body spines. Forebody 280-546 long or 26-37% of BL; hindbody 520-976 long or 50-64% of BL. Prepharynx well defined, 206-574 long. Pharynx thick-walled, muscular, usually pyriform, located with anterior margin extends beyond anterior margin of ventral sucker to completely posterior to ventral sucker, 69-112 long, 66-126 wide; prepharynx 2.8-8.3 times longer than pharynx, with posterior prepharynx and pharynx surrounded by few gland cells of probable of digestive nature. Oesophagus not as well defined as prepharynx, often folded slightly among other organs, 39-188 long. Caecum in hindbody, lined with large cells, with posterior end having

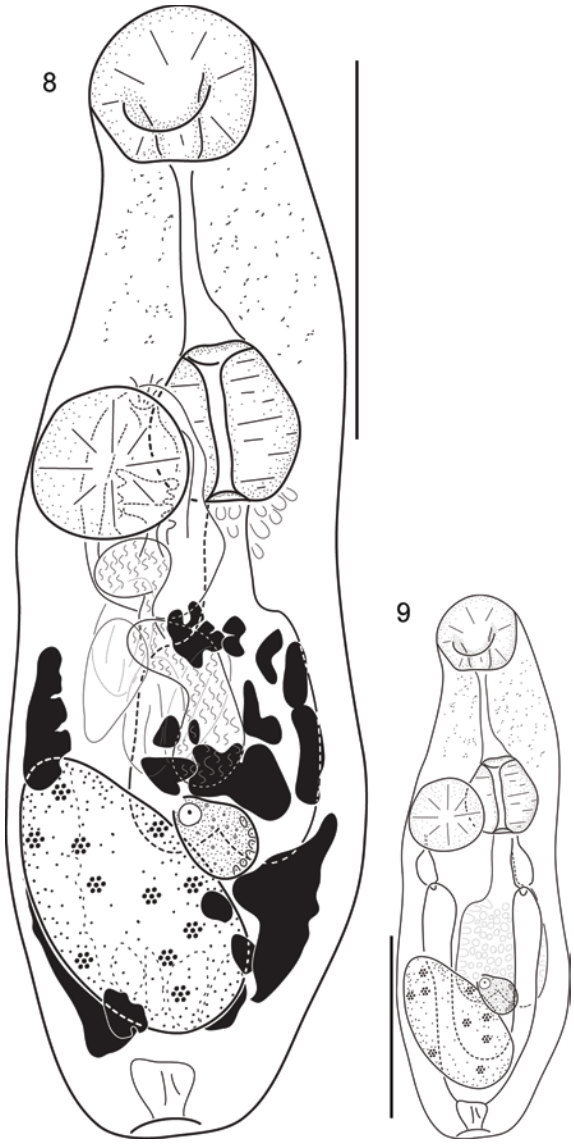


Figures IV.4-7. Malabarotrema n.sp.1 4. ventral wholemount; 5. ventral wholemount; *Scale-bars* 4, 250 µm; 5, 250µm; 6,7 100 µm. 6. ovarian complex of specimen killed while under coverslip pressure; 7. hermaphroditic sac of specimen killed while under coverslip pressure;

slightly indented, 212-354 long, 108-240 wide, commencing 340-820 from anterior end of body or 35-53% of BL; post-caecal space 125-495 or 14-32% of BL.

Testis medial, with anterior margin straight or beveled, with posterior end rounded, overlapping posterior end of caecum to 74 distant, 195-520 from ventral sucker, 182-377 long, 95-214 wide; post-testicular space 49-240 or 4-15% of BL. Hermaphroditic sac dorsal to ventral sucker, ventral to pharynx, 171-245 long, 82-121 wide, terminating posterior to ventral sucker; sac containing following structures (internal seminal vesicle 70-107 long, 41-65 wide; male duct thin-walled; prostatic bulb prominent, with size dependent on amount of sperm and eggs present; male duct joining female duct in posterior half of sac); hermaphroditic duct strongly muscularized, posterior portion containing spirally arranged muscular pads. External seminal vesicle sac-like, 68-269 long, 32-60 wide. Genital atrium elongate laterally, without evidence of scleritization, genital pore about at level of anterior margin of ventral sucker in crease between elevated ventral sucker and plane of forebody.

Ovary medial, ventral to caecum, situated laterally along anterior margin ventral to testis, 79-137 long, 44-83 wide, 145-461 from ventral sucker. Mehlis' gland anterior to ovary. Laurer's canal dorsal to testis, ventral to caecum; pore dorsal, near mid-testicular level. Vitellarium consisting of numerous areas follicles, commencing near level of posterior margin of ventral sucker to 43 posterior, evenly distributed dorsally, ventrally not confluent; ventral to testis or uterus, confluent posterior to testis. Uterus direct from ovary to hermaphroditic sac, without loops; proximal portion often filled with sperm; metraterm lacking. Eggs thin-shelled, 1-15 in number (from 20 ovigerous specimens), 68-88 long, 41-67 (averaging 78 X 54) wide when measured from distal end of uterus, with incompletely developed miracidium lacking eye-spots.



Figures IV.8-9. Unisaccoides vitellosus. 8. ventral wholemount. 9. ventral wholemount showing extent of excretory vesicle. *Scale-bars* 200µm.

Lymphatic tubes in forebody. Excretory vesicle Y-shaped, dorsal to testis, bifurcating near mid level of testis, terminating near anterior margin of ovary; pore terminal.

Remarks

Malabarotrema n. sp. 1 differs from *M. lobolecithum* by the presence of spines between the oral and ventral suckers, the lack of enlarged spines dorsal to the oral sucker,

slightly smaller eggs, and molecular differences. *Malabarotrema* n. sp. 1 differs from *M. megaorchis* by the more elongate body, having slightly larger eggs, and molecular differences.

Unisaccodies Martin, 1973

Diagnosis: Body pyriform to fusiform. Eye-spot pigment diffuse. Oral sucker terminal to sub-terminal. Prepharynx distinct. Pharynx relatively long. Oesophagus longer or shorter than prepharynx. Caecum sac-like, longer than wide, with or without indentation posteriorly. Hermaphroditic sac sac-like to arcuate about twice as long as wide. Hermaphroditic duct long, unarmed, with spirally arranged pads posteriorly, with distal end smooth and muscular. Female duct short. Metraterm absent. External and internal seminal vesicle present. Prostatic bulb distinct. Genital pore circular. Testis spherical to ellipsoidal in posterior half of body. Uterus restricted to between testis and hermaphroditic sac. Vitellarium numerous small groups of follicles occupying most of hindbody. Miracidium in utero underdeveloped, lacking eye-spots. Excretory vesicle Y-shaped, reaching anterior to testis; pore terminal, with muscular sphincter. In intestine of Mugilidae from Indo-West Pacific region. Type-species *Unisaccoides vitellosus* Martin, 1973.

The type-species of *Unisaccoides* had previously been allocated to *Skrjabinolecithum* by Overstreet and Curran (2005) but is resurrected herein.

Unisaccoides vitellosus Martin, 1973

Syns Skrjabinolecithum vitellosum (Martin, 1973) Overstreet and Curran, 2005.

Type-host: *Mugil cephalus* Linnaeus, flathead grey mullet (Mugilidae).

Other host: *Liza argentea* (Quoy & Gaimard), flat-tail mullet (Mugilidae) by Martin, 1973b); *Chelon subvirdis* (Valenciennes), greenback mullet (Mugilidae) (this chapter)

Type-locality: Brisbane River, Brisbane, Queensland, Australia.

Site of infection: intestine.

Material examined: *ex. Chelon subvirdis*; Queensland, Australia: Beelbi Creek (25° 15'S 152° 40'E) Coll. Pulis, Andres March 2010, Fishing Creek (25° 57'S 150° 47'E) Coll. Pulis, Andres March 2010.

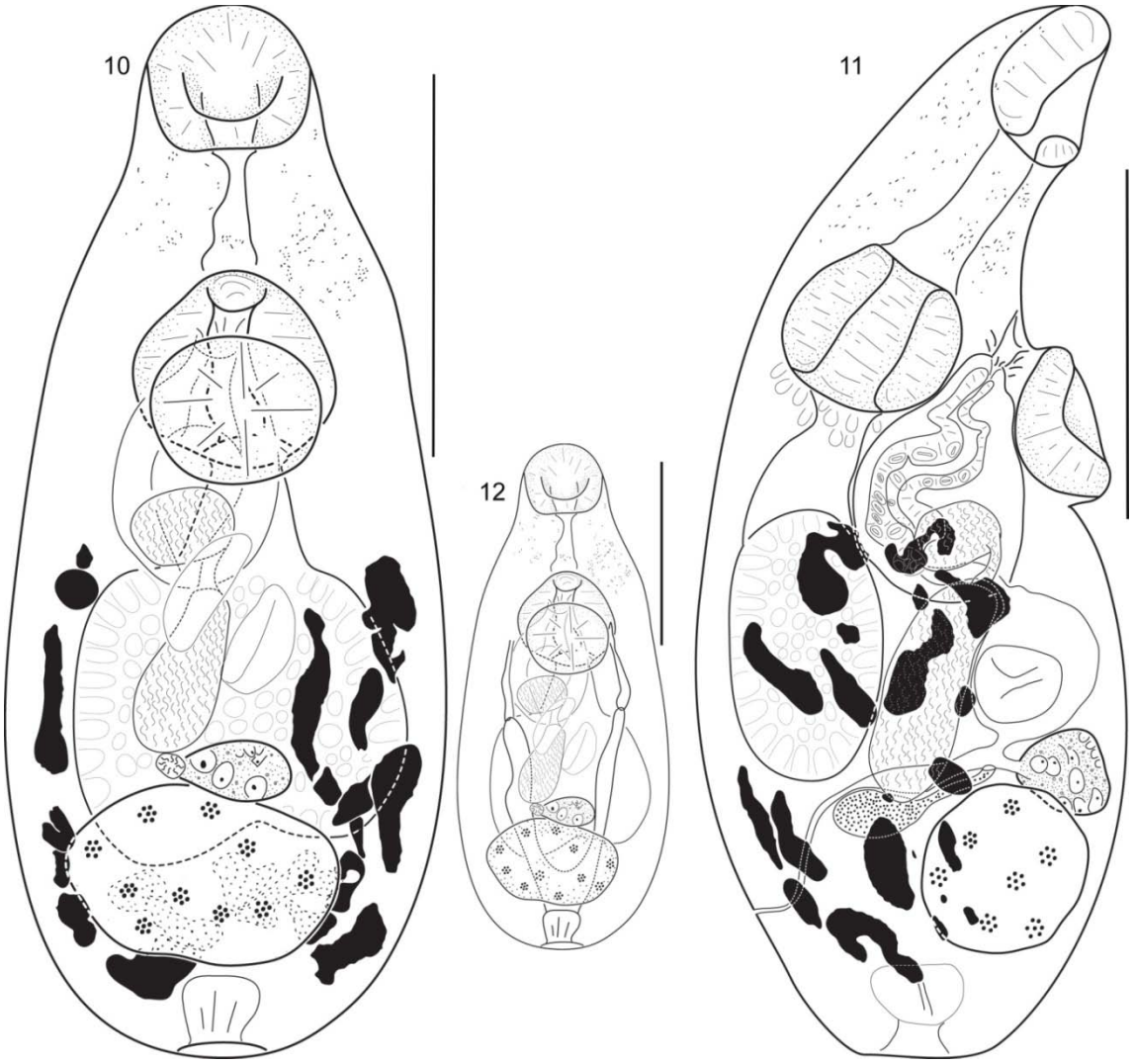
Specimens Deposited: USNPC NTM BM TBD, representative sequence, EP-379. Holotype: No. 716 Allen Hancock Parasitology Collection, University of Southern California, but current whereabouts unknown; unsuccessful attempts were made to borrow specimens from University of Southern California Museum, Santa Barbara Museum of Natural History, and Los Angeles County Museum of Natural History (Pers. comm. Daniel Geiger SBMNH, Jody Martin NHMLAC).

Description: Measurements based on 8 wholemounted specimens. Body fusiform, 547-805 long, 171-287 wide, widest in hindbody, width 31-41% of BL. Tegment spined over entire body; spines becoming smaller and more sparse posteriorly. Eye-spot pigment dispersed in forebody. Oral sucker subterminal, 64-84 long, 68-96 wide, with mouth opening anteroventrally. Ventral sucker slightly elevated, 65-87 long, 73-96 wide, covered by spines, ventral sucker 97-115% longer than oral sucker, 97-107% wider than oral sucker; spines covering ventral sucker smaller than body spines, similar in shape. Forebody 177-244 long or 28-33% of BL; hindbody 304-479 long or 50-60% of BL. Prepharynx 81-145 long. Pharynx thick-walled, located dorsal to ventral sucker, 66-86

long, 60-93 wide, with posterior portion surrounded by few gland cells probably of digestive nature; prepharynx 1.2-1.7 pharyngeal lengths. Oesophagus 61-95 long, 54-80% of prepharynx length. Caecum in hindbody, lined with large cells, with posterior end slightly indented, 96-169 long, 74-123 wide, commencing 255-410 from anterior end of body or 28-33% of BL; post-caecal space 146-243 or 25-32% of BL.

Testis commencing near posterior level of caecum, 112-275 from ventral sucker, shape rounded to slightly elongate, transverse to longitudinal, 99-215 long, 89-148 wide; post-testicular space 32-87 or 5-16% of BL. Hermaphroditic sac dorsal to ventral sucker, 105-149 long, 68-88 wide, terminating posterior to ventral sucker; sac containing following structures (internal seminal vesicle long, narrow, 31-57 long, 17-30 wide; male thin-walled; prostatic bulb prominent, with size dependent on amount of sperm and eggs present; male duct joining female duct near mid-length of sac); hermaphroditic duct muscularised, posterior portion containing series of spirally arranged pads without evidence of scleritization. External seminal vesicle sac-like, terminating near ovarian level, 91-102 long, 30-37 long. Genital pore medial, round, immediately anterior to ventral sucker.

Ovary medial, ventral to caecum, contiguous with testis, 44-70 long, 38-58 wide, 95-218 from ventral sucker. Mehlis' gland anterior to lateral to ovary. Laurer's canal present; pore to external not observed. Vitellarium tubes or round, in hindbody, ventrally not confluent; follicles present dorsal to testis, terminating posterior to testis. Uterus confined to area anterior to testis and posterior to hermaphroditic sac; proximal portion often filled with sperm; metraterm lacking. Eggs, 3-32 in number, 58-76 long, 27-42



Figures IV.10-12. Unisaccoides n. sp. 1 10. Ventral wholemount; 11. Lateral wholemount;12. Ventral wholemount showing extent of excretory vesicle. *Scale-bars* 10-12, 200µm.

wide (average 71 X 33) when measured from distal end of uterus, with incompletely developed miracidium lacking eye-spots.

Excretory vesicle Y-shaped, dorsal to testis, bifurcating near posterior testis, terminating near ventral sucker region; pore terminal.

Remarks

The four specimens described by Martin (1973b) were slightly smaller than my specimens, and only one was ovigerous. My series of eight specimens provided a wider variation in measurements. I did not observe spines on the pads in the hermaphroditic duct reported by Martin (1973b).

Unisaccoides n. sp. 1

Type-host: *Chelon macrolepis* (Smith), largescale mullet (Mugilidae).

Other hosts: *Liza subvirdis* (Valenciennes), greenback mullet (Mugilidae).

Type-locality: Western Australia, Australia: Withnell Bay (20° 35'S 116° 47'E) Feburary 2010 Coll. Pulis, Andres.

Other localities: *ex. Chelon macrolepis* Western Australia, Australia: creek south of Learmouth (22° 14'S 114° 07'E) February 2010 Coll. Pulis, Andres. *ex. Liza subvirdis* Western Australia, Australia, Barred Creek (17° 40'S 122° 12'E) February 2010 Coll. Pulis, Andres.

Site of infection: intestine.

Type-material: USNPC NTM BM TBD, representative sequence, EP-077

Description: Figures 10-11, 20. Tables 1-3.

Measurements based on 13 wholemounted specimens. Body fusiform, 498-649 long, 189-269 wide, widest in hindbody, width 37-47% of body length. Tegument spined over entire body; spines becoming smaller and more sparse posteriorly. Eye-spot pigment dispersed in forebody. Oral sucker subterminal, with mouth opening anteroventrally, 71-95 long, 75-98 wide. Ventral sucker slightly elevated, 67-100 long, 75-104 wide, length 91-116% of oral sucker length; width 92-109% of oral sucker width, covered by spines;

spines on ventral sucker smaller than body spines, similar in shape. Forebody 151-208 long, or 26-35% of BL; hindbody 257-362 long, or 47-57% of BL. Prepharynx 45-90 long. Pharynx thick-walled, dorsal to ventral sucker or further posterior, 75-102 long, 85-109 wide, surrounded posteriorly by a few gland cells probably of digestive nature; prepharynx; 0.4-1.0 times longer than pharynx. Esophagus 44-87 long, 60-114% of prepharynx length. Caecum lined with large cells, posterior end slightly indented, 120-179 long, 119-184 wide, commencing 256-334 from anterior end, or 45-58% of BL; post-caecal space 84-188, or 15-29% of BL.

Testis variously positioned from parallel to perpendicular to plane of body, usually oblique, commencing at level of posterior of caecum; anteriormost portion usually sinestral, spherical to bean shaped, 87-171 long, 70-179 wide, 66-176 from ventral sucker; post-testicular space 43-77 or 8-12% of BL. Hermaphroditic sac dorsal to ventral sucker, 129-155 long, 77-99 wide, terminating posterior to ventral sucker; sac containing the following (internal seminal vesicle long, narrow, 34-56 long, 23-43 wide; male duct thin-walled; prostatic bulb prominent with size dependent on amount of sperm and eggs present; male duct joining female duct near middle of sac); hermaphroditic duct muscularized, with posterior portion containing series of pads; pads without evidence of scleritization. External seminal vesicle long, usually directed posteriorly, terminating near ovarian level, 45-121 long, 24-54 wide. Genital pore medial, immediately anterior to ventral sucker.

Ovary ventral to caecum, contiguous with testis, usually dextral, 37-67 long, 30-50 wide, 91-159 from ventral sucker. Mehlis' gland anterior to lateral to ovary. Laurer's canal present; pore dorsal to testis. Vitellarium arranged lateral and dorsal to testis,

ventrally not confluent, terminating posterior to testis, small round follicles dorsally, tubes lateral to testis. Vitelline duct wide, ventral to testis and ovary. Uterus confined to area anterior to testis and posterior to hermaphroditic sac; proximal portion often filled with sperm; metraterm lacking. Eggs, 1-15 in number, 60-75 long, 23-46 wide (averaging 66 X 34) when measured from distal portion of uterus, with incompletely developed miracidium, not exhibiting eye-spots.

Excretory vesicle Y-shaped, dorsal to testis, bifurcating near posterior testis, terminating near ventral sucker region; pore terminal.

Remarks

Unisaccoides n. sp 1 differs from the slightly larger, trimmer *Unisaccoides vitellous* by having a larger pharynx, and being located in Western Australia rather than Queensland.

Unisaccus Martin, 1973

Syn Pseudounicoelium Ahmad, 1987

Body pyriform to fusiform. Eye-spot pigment diffuse. Oral sucker terminal to sub-terminal. Prepharynx relatively long. Pharynx present. Oesophagus longer or shorter than prepharynx. Caecum sac-like, longer than wide, with or without indentation posteriorly. Hermaphroditic sac sac-like about twice as long as wide. Hermaphroditic duct long, with spirally arranged pads posteriorly, with distal end smooth and highly muscular. Female duct short. Metraterm absent. External and internal seminal vesicle present. Prostatic bulb distinct. Testis spherical to ellipsoidal in posterior half of body. Uterus in hindbody, occasionally extending into post-testicular space. Vitellarium restricted to lobes of follicles lateral to area between ventral sucker and testicular space.

Eggs thin-shelled; mature distal eggs containing well-developed miracidium with eye-spots. Excretory vesicle Y-shaped, reaching anterior to testis; pore terminal, with muscular sphincter. In intestine of Mugilidae in Indo-West Pacific Region. Type-species: *Unisaccus brisbanensis* Martin, 1973.

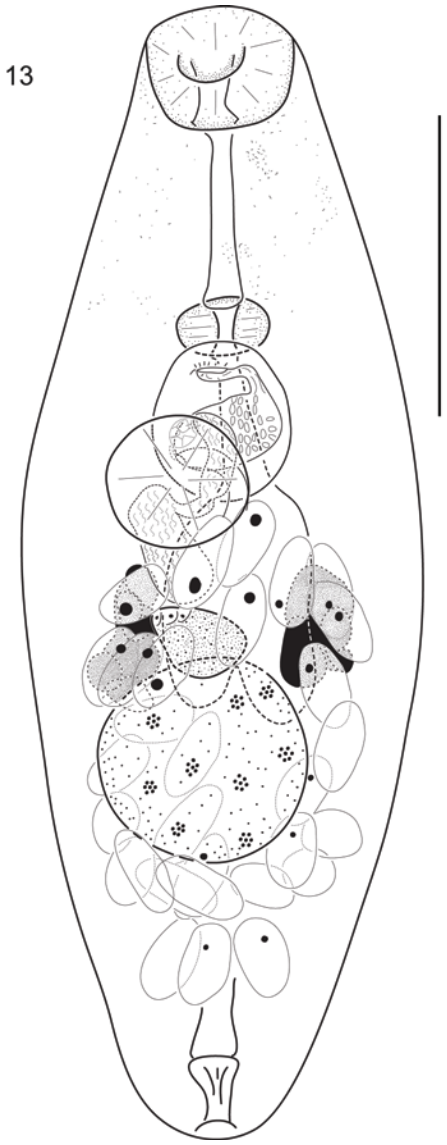


Figure IV.13. Unisaccus brisbanensis, ventral view of wholemount; *scale-bar* 200µm.

Unisaccus brisbanensis Martin, 1973

Type-host: *Mugil cephalus* Linnaeus, 1758, flathead grey mullet (Mugilidae).

Other hosts: *Chelon subvirdis* (Valenciennes), greenback mullet (Mugilidae).

Type-locality: Brisbane River, Brisbane, Queensland, Australia

Site of infection: Intestine

Material examined: ex. *Chelon subvirdis*: Queensland, Australia: Fishing Creek (22° 57'S 150° 47'E) Coll. Pulis, Andres March 2010, Causeway Lake Outlet (23° 12'S 150° 47'E) Coll. Pulis, Andres March 2010, boat ramp near Keppel Sands (23° 19'S 150° 47'E) Coll. Pulis, Andres March 2010, Beelbi Creek (25° 15'S 152° 40'E) Coll. Pulis, Andres March 2010, Eli Creek (25° 16'S 152° 49'E) Coll. Pulis, Andres March 2010.

Specimens Deposited: Holotype: No. 715 Allen Hancock Parasitology Collection, University of Southern California, but current whereabouts unknown; unsuccessful attempts were made to borrow specimens from University of Southern California Museum, Santa Barbara Museum of Natural History, and Los Angeles County Museum of Natural History (Pers. comm. Daniel Geiger SBMNH, Jody Martin NHMLAC). USNPC NTM BM-TBD, representative sequence, EP-376.

Description: Figures 13-20, Tables 1-3.

Measurements based on 13 wholemount specimens. Body fusiform, 519-919 long, 186-382 wide, widest in hindbody, width 31-42% of BL. Tegument spined over entire surface, spines becoming smaller and more sparse posteriorly; spines hastate, about 4-6 long (measured from forebody), directed posteriorly. Eye-spot pigment dispersed in forebody. Oral sucker subterminal, 63-92 long, 68-130 wide, with mouth opening anteroventrally. Ventral sucker slightly elevated, 56-108 long, 63-120 wide, ventral sucker 89-126% longer than oral sucker; 76-117% wider than oral sucker, covered by spines; spines on ventral sucker smaller than body spines, similar in shape. Forebody 181-320 long or 33-41% of BL; hindbody 251-503 long or 45-60% of BL. Prepharynx

well defined, 61-160 long. Pharynx anterior to level of ventral sucker, 33-51 long, 51-67 wide; prepharynx 1.8-3.8 times longer than pharynx. Oesophagus 63-137 long, terminating near level of posterior ventral sucker. Caecum mostly in hindbody, lined with large cells, posterior end having indentation, 109-234 long, 55-179 wide, commencing 201-394 from anterior end of body or 33-50% of BL; post-caecal space 149-332 or 28-47% of BL.

Testis medial, round, overlapping posterior end of caecum, 14-100 from ventral sucker, 97-184 long, 88-173 wide; post-testicular space 108-231 or 18-29% of BL. Hermaphroditic sac dorsal to ventral sucker, ventral to pharynx, 95-161 long, 79-113 wide, terminating near posterior margin of ventral sucker; sac containing following structures (internal seminal vesicle 51-93 long, 14-27 wide; male duct thin-walled; prostatic bulb prominent, with size dependent on amount of sperm and eggs present; male duct joining female duct in posterior half of sac); hermaphroditic duct strongly muscularized, posterior portion containing spirally arranged muscular pads. External seminal vesicle sac-like, 60-116 long, 28-41 wide. Genital pore noticeably anterior to ventral sucker.

Ovary medial, ventral to caecum and testis, contiguous with anterior portion of testis, 36-64 long, 32-48 wide, 20-80 from ventral sucker. Mehlis' gland anterior to ovary. Laurer's canal dorsal to testis, ventral to caecum; pore dorsal, near mid-testicular level. Vitellarium consisting of two areas of follicles lateral to ovary, about 6-10 follicles in each group. Uterus extensive, with numerous eggs, with loops descending posterior to testis; proximal portion often filled with sperm; metraterm lacking. Eggs thin-shelled, 4-

>70 in number, 50-66 long, 23-33 wide (average 58 X 29) when measured from distal end of uterus; maturing distal eggs containing miracidium with eye-spots.

Lymphatic tubes in forebody.Excretory vesicle Y-shaped, dorsal to testis, bifurcating near posterior testis, terminating near ventral sucker region; pore terminal.

Remarks

Other than the published values for prepharynx length and ovary, which appear to have a typographical error, my specimens agree with those described by Martin (1973b). Based on scale-bar associated with Figure 1 (Martin, 1973b), the ovarian diameter is about 40 rather than 18-22, and the oesophagus is about 130 rather than 170-280; other values presented by Martin agreed with his Figure 1.

Unisaccus lizae (Liu, 2002) n. comb.

Syn Saccocoelioides lizae Liu, 2002.

Type-host: *Liza carinata* (Valenciennes), kelled mullet (Mugilidae), reported as *Liza carinatus* (Cuvier et Valenciennes) by Liu (2002).

Other host: *Mugil cephalus*, Linnaeus, 1758, flathead grey mullet (Mugilidae).

Type-locality: Jimei, Xiamen (24° 25' 24" ~ 33' 14" N, 118° 03' 58" ~ 11' 48" E), Fujian, China.

Other-locality: ex. *Mugil cephalus* purchased at fish market, Daya Bay (22° 43'N 114° 32'E) March 2009, Guangdong Province, China.

Site of infection: Intestine.

New-material: ex. *Mugil cephalus* USNPC-TBD, represenative sequence, EP-640

Description. (Figures 14, 20 Tables 1-3).

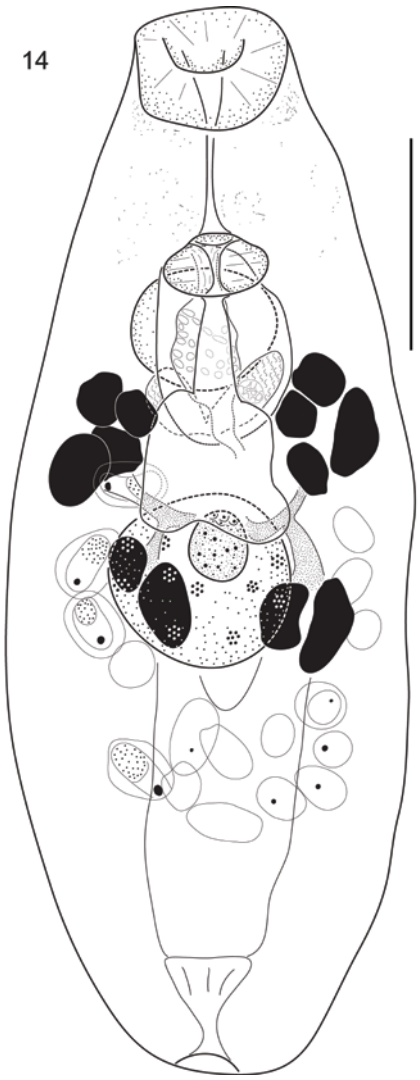


Figure IV.14. Unisaccus lizae, dorsal vie wholemount. *Scale-bar* 200µm.

Measurements based on 6 wholemounted specimens. Body fusiform, 697-964 long, 287-382 wide, widest in hindbody, width 35-49% of BL. Tegument spined over entire surface, spines becoming smaller and more sparse posteriorly; spines hastate, about 4-6 long (measured from forebody), directed posteriorly. Eye-spot pigment dispersed in the forebody. Oral sucker subterminal, 87-125 long, 102-129 wide, with mouth opening anteroventrally. Ventral sucker slightly elevated, 103-120 long, 107-139 wide, 82-126% longer than oral sucker length; 85-124% wider than oral sucker, covered by spines; spines

on ventral sucker smaller than body spines, similar in shape. Forebody 217-277 long or 23-36% of BL; hindbody 345-625 long or 49-65% of BL. Prepharynx 47-134 long. Pharynx near anterior margin of ventral sucker, 51-76 long, 69-102 wide; prepharynx 0.6-2.5 times longer than pharynx. Posterior of prepharynx and pharynx surrounded by few gland cells probably of digestive nature. Oesophagus 67-110 long, terminating near posterior margin of ventral sucker. Caecum in hindbody, lined with large cells, posterior end having indentation, 130-189 long, 106-130 wide, commencing 308-386 from anterior end of body or 38-45% of BL; post-caecal space 207-483 or 30-50% of BL.

Testis medial, rounded, overlapping posterior end of caecum, 52-130 from ventral sucker, 116-117 long, 103-181 wide; post-testicular space 180-373 or 26-39% of BL. Hermaphroditic sac dorsal to ventral sucker, 123-177 long, 86-128 wide, terminating near posterior margin ventral sucker; sac containing following structures (internal seminal vesicle 51-81 long, 26-39 wide; male duct thin-walled; prostatic bulb prominent, with size dependent on amount of sperm and eggs present; male duct joining female duct in posterior half of sac); hermaphroditic duct strongly muscularized, with posterior portion containing spirally arranged pads of uncertain function. External seminal vesicle sac-like, 66-88 long, 36-55 wide. Genital pore immediately anterior to ventral sucker.

Ovary ventral to caecum and testis, contiguous with testis, 51-79 long, 49-55 wide, 39-103 from ventral sucker. Mehlis' gland anterior to ovary. Laurer's canal dorsal to testis, ventral to caecum; pore dorsal, testicular level. Vitellarium consisting of two areas of follicles lateral to ovary, about 6-8 follicles each side, with anterior and posterior group, configuration essentially H-shaped in most specimens including vitelline duct and reservoir. Uterus extensive, with numerous eggs loops descending posterior to testis;

proximal portion often filled with sperm; metraterm lacking. Eggs thin-shelled, 63-78 long, 30-50 wide (average 70 X 40) when measured from distal end of uterus, maturing distal eggs containing well developed miracidium with eye-spots.

Lymphatic tubes in forebody.Excretory vesicle Y-shaped, dorsal to testis, bifurcating near posterior testis, terminating near ventral sucker region; pore terminal.

Remarks

The specimens of Liu (2002) appear to have been killed while under coverslip pressure; those measured here were killed by addition of hot water. *Unisaccus lizae* can be differentiated from other species in the genus by oral and ventral suckers nearly equal in size, ovary smaller than testis, and vitellarium in four goups of follicles.

Unisaccus n. sp. 1

Type-host: *Chelon planiceps* (Valenciennes), tade grey mullet (Mugilidae) keyed to *Liza planiceps* in FAO guide (Harrison & Senou, 1997) and tentatively identified as *Moolgarda ordensis*, which is currently a synonym of *Liza alata* (Steindachner) diamond mullet (Mugilidae) (did not posses falcate fins), (pers. com. Rex Williams of the Northern Territory Museum). I collected tissue samples from three fish that keyed to *L. planiceps*, and produced partial COI sequences for these specimens. Unfortunately, these sequences most likely represent 2 species of mullet, neither of which is *L. planiceps* (*sensu stricto*). One fish from Ludmilla Creek (666) and one from Buffalo Creek (671) are 99% similar to GenBank sequence JQ060429 of a vouchered fish (NTMS.15537-001) identified as *Chelon planiceps* from Northern Territory Australia (Durand, Shen et al., 2012) and suggested as *Planiliza* sp. F in Durand Chen et al. (2012). One fish from Buffalo Creek (670) was 97% similar to JQ060488 identified as *Liza* sp. collected at Pangasinan,

Philippines suggested as *Planilizae* sp. C by Durand, Chen, et al., (2012). The problem is that the fish sequenced were not the specimens I obtained *Unisaccus* n. sp. 1 from, but without the benefit of the recent sequencing of mugilids by Durand Shen et al. (2012) I would have simply listed the host as *L*. *planiceps*.

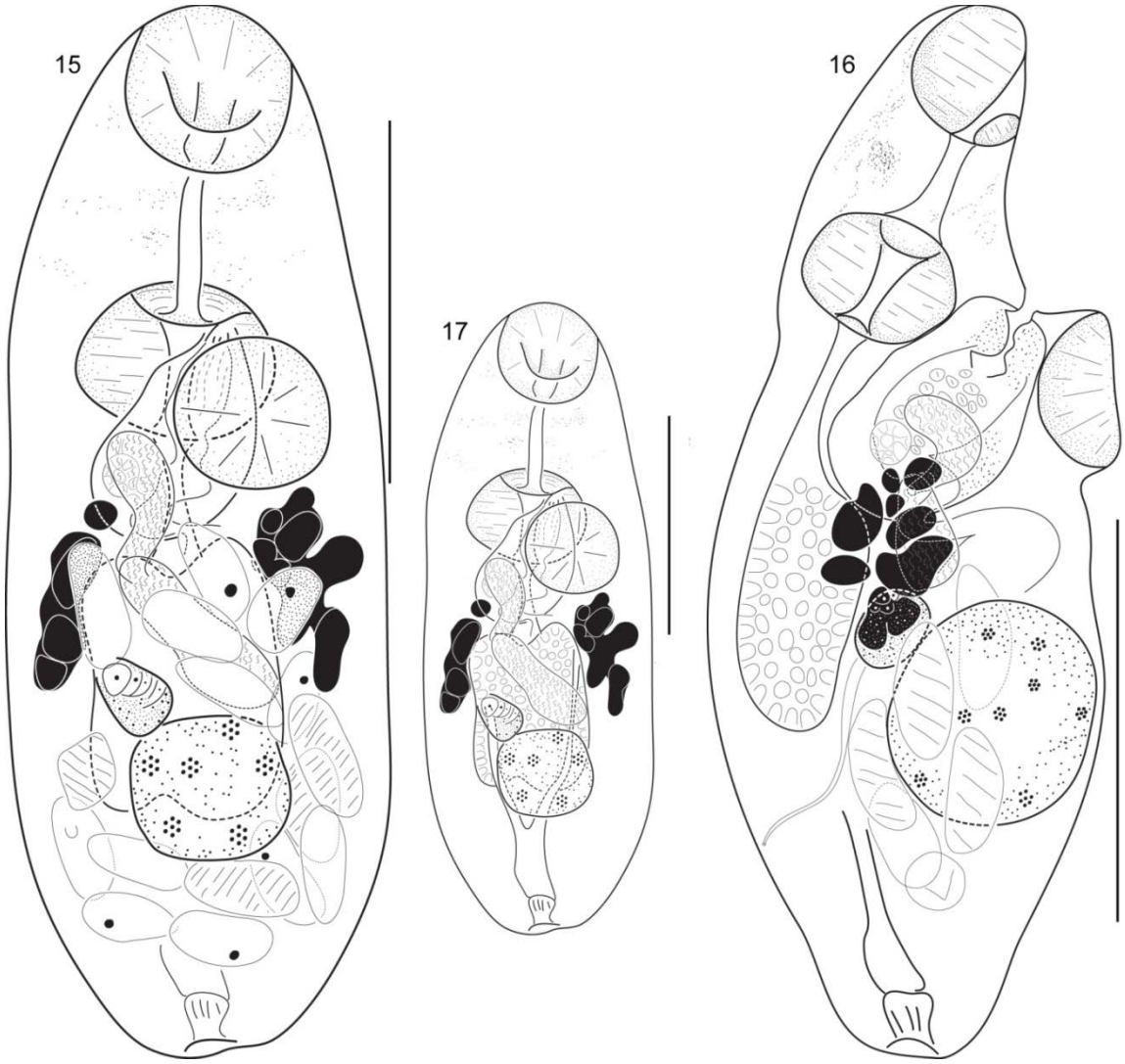
Type-locality: Northern Territory, Australia; Ludmilla Creek (12° 25'S 130° 50'E) Coll. Pulis, Andres March 2010, Buffalo Creek (12° 12'S 130° 55'E) Coll. Pulis, Andres March 2010.

Site of infection: Intestine.

Type-material: USNPC NTM BM-TBD, representative sequence, EP-277

Description: Figures 15-17, 20 Tables 1-3.

Measurements based on 6 wholemount specimens. Body fusiform, 514-647 long, 194-256 wide, widest in hindbody, width 34-42% of BL. Tegument spined over entire surface, spines becoming smaller and more sparse posteriorly; spines hastate, about 6-7 long (measured from forebody), directed posteriorly. Eye-spot pigment dispersed in the forebody. Oral sucker subterminal, 66-87 long, 71-94 wide, with mouth opening anteroventrally. Ventral sucker slightly elevated, 73-87 long, 72-90 wide; 96-112% longer than oral sucker, 93-103% wider than oral sucker, covered by spines, ventral sucker spines smaller than body spines, similar in shape. Forebody 140-191 long or 27-33% of BL; hindbody 291-359 long or 53-57% of BL. Prepharynx 36-86 long. Pharynx dorsal to ventral sucker, 65-92 long, 84-112 wide; prepharynx 0.6-0.9 times longer than pharynx. Posterior prepharynx and pharynx surrounded by few gland cells probably of digestive nature. Oesophagus 60-88 long, terminating near posterior margin of ventral sucker. Caecum located in hindbody, lined with large cells, posterior end having



Figures IV.15-17. Unisaccus n. sp.1 15. Ventral view of wholemount; 16. Lateral view of wholemopunt; 17. Lateral view of wholemount without eggs showing excretory vesicle. *Scale-bars* 15-17, 200µm.

indentation, 116-168 long, 60-127 wide, commencing 238-306 from anterior end of body or 42-52% of BL; post-caecal space 116-176 or 20-28% of BL. Testis medial, rounded, overlapping posterior end of caecum, 84-116 long, 91-117 wide, 73-152 from ventral sucker; post-testicular space 82-123 or 15-21% of BL. Hermaphroditic sac dorsal to ventral sucker, ventral to pharynx, 104-132 long, 63-90 wide, terminating near posterior to ventral sucker; sac containing following structures (internal seminal vesicle sac-like,

50-68 long, 21-31 wide; male duct thin-walled; prostatic bulb prominent, with size dependent on amount of sperm and eggs present; male duct joining female duct in posterior half of sac); hermaphroditic duct strongly muscularized, with posterior portion containing spirally arranged muscular pads; pads without evidence of scleritization. External seminal vesicle sac-like, 52-107 long, 25-41 wide. Genital pore immediately anterior to ventral sucker.

Ovary medial, ventral to caecum and testis, usually contiguous with testis, 43-61 long, 27-50 wide, 70-103 from ventral sucker. Mehlis' gland anterior to ovary. Laurer's canal passes between testis and caecum; pore dorsal. Vitellarium consisting of two areas of follicles lateral space between ventral sucker and testis, about 8-10 follicles in each group. Uterus extensive specimens with numerous eggs, uterine loops posterior to testis; proximal portion often filled with sperm; metraterm lacking. Eggs thin-shelled, 6-26 in number, 68-83 long, 26-36 wide (average 76 X 31) when measured from distal end of uterus, maturing distal eggs containing miracidium with eye-spots.

Lymphatic tubes in forebody. Excretory vesicle Y-shaped, dorsal to testis, bifurcating near posterior portion of testis, terminating near ventral sucker region; pore terminal.

Remarks

Unisaccus n. sp 1 can be differentiated from the other members of *Unisaccus* by having the testis larger than ovary, oral sucker about the same size as the ventral sucker, and the prepharynx shorter than the pharynx.

Unisaccus n. sp. 2

Type-host: *Chelon macrolepis* (Smith), largescale mullet (Mugilidae).

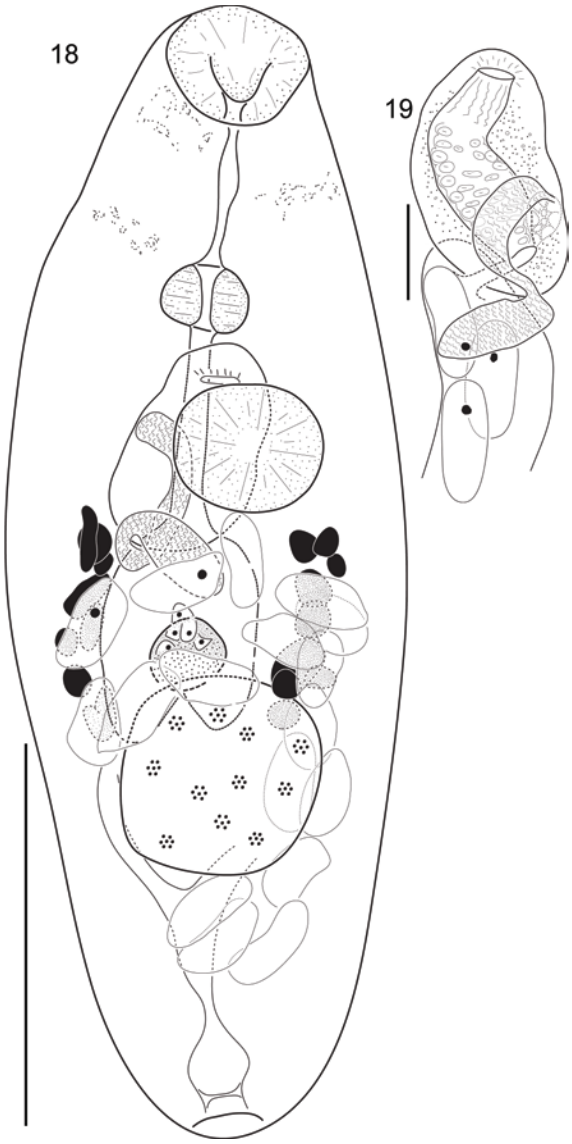
Type-locality: Western Australia, Australia; Withnell Bay (20° 35'S 116° 47'E) February 2010 Coll. Pulis, Andres, creek mouth south of Learmouth airport (22° 14'S 114° 07'E) February 2010 Coll. Pulis, Andres.

Site of infection: Intestine.

Type-material: USNPC NTM BM GenBank.

Description: Figures 18-20, Tables 1-3.

Measurements based on 9 wholemount specimens. Body fusiform, 451-591 long, 158-202 wide, widest in hindbody, width 32-38% of BL. Tegument spined over entire surface, spines becoming smaller and more sparse posteriorly; spines hastate, about 4-6 long (measured in forebody), directed posteriorly. Eye-spot pigment dispersed in forebody. Oral sucker subterminal, 62-72 long, 58-85 wide, mouth opening anteroventrally. Ventral sucker slightly elevated, 62-90 long, 62-88 wide, 89-134% longer than oral sucker, 89-108% wider than oral sucker, covered by spines; spines covering ventral sucker smaller than body spines; similar in shape. Forebody 168-199 long or 31-39% of BL; hindbody 212-329 long or 47-56% of BL. Prepharynx 43-96 long. Pharynx anterior to ventral sucker, 31-46 long, 46-96 wide; prepharynx 1.2-2.8 times longer than pharynx. Oesophagus 51-98 long, terminating near posterior margin of ventral sucker. Caecum in hindbody, lined with large cells, posterior end having slight indentation, 87-148 long, 63-100 wide, commencing 178-272 from anterior end of body or 31-39% of BL; post-caecal space 146-211 or 28-38% of BL.



Figures IV.18-19. Unisaccus n. sp. 2. 18. ventral view of wholemount; 19. terminal genitailia *Scale-bars* 18, 200µm; 19, 50µm.

Testis medial, rounded, overlapping posterior end of caecum, 21-107 from ventral sucker, 86-128 long, 74-110 wide; post-testicular space 70-141 or 14-26% of BL. Hermaphroditic sac dorsal to ventral sucker, ventral to pharynx, 90-128 long, 70-88 wide, terminating posterior to posterior margin of ventral sucker; sac containing following structures (internal seminal vesicle 45-61 long, 16-40 wide; male duct thin-walled; prostatic bulb prominent, with size dependent on amount of sperm and eggs present; male

duct joining female duct in posterior half of sac); hermaphroditic duct strongly muscularized, posterior portion containing spirally arranged muscular pads; pads without evidence of scleritization. External seminal vesicle sac-like, 32-74 long, 14-33 wide. Genital pore medial, immediately anterior to ventral sucker.

Ovary medial, ventral to caecum, contiguous with testis to 35 anterior, 27-44 long, 22-46 wide, 11-80 from ventral sucker. Mehlis' gland anterior to ovary. Laurer's canal dorsal to testis, ventral to caecum; pore dorsal, near mid-testicular level. Vitellarium consisting of two areas of follicles lateral to space between testis and ventral sucker, about 6-10 follicles in each group. Uterus extensive with numerous eggs, with loops descending posterior to testis; proximal portion often filled with sperm; metraterm lacking. Eggs thin-shelled, 5-18 in number, 51-65 long, 19-29 wide (average 56 X 24) when measured from distal end of uterus, maturing distal eggs containing miracidium with eye-spots.

Excretory vesicle Y-shaped, dorsal to testis, bifurcating near posterior testis, terminating near ventral sucker region; pore terminal.

Species *incertae sedis*

Unisaccus sprenti (Martin, 1973) Blasco-Costa, Montero, Gibson, Balbuena, Raga, and Kostadinova, 2009

Syns Lecithobotrys sprenti Martin, 1973; *Saccocoelium sprenti* (Martin, 1973) Overstreet and Curran, 2005.

I agree with Overstreet and Curran (2005) that *Lecithobotrys* is not the proper placement for *Unisaccus sprenti* and with Blasco-Costa, Montero, Gibson, Balbuena, Raga and Kostadinova (2009) that *Saccocoelium* is not a better repository. I cannot

confidently accept *Unisaccus sprenti* in *Unisaccus*. Figure 24 by Martin (1973a) illustrated what appears to be a caecum and does not show an oesophagus or indentation posteriorly (other Australian species are indented at the posterior portion of the caecum). In the description of *Unisaccus sprenti*, Martin used the term "ceca" (Page 93) rather than cecum, and, in the following papers dealing with species possessing an undivided caecum, he did not transfer or otherwise address the generic affiliation of *Unisaccus sprenti* (Martin, 1973b; 1973c). Martin (1973a) also found a second type of cercaria that he believed to be that of *Unisaccus sprenti*. Both of his figures of that cercaria (Martin, 1973a; Figure 6) and metacercaria (Martin, 1973a; Figure 18), clearly showed the two caeca. Had there been only one caecum and if *Unisaccus sprenti* truly has an undivided caecum, this feature should have eliminated the larval forms from consideration as conspecific with *Unisaccus sprenti*. Additionally, the pads of the hermaphroditic duct in *Unisaccus sprenti* are figured (Martin, 1973a; Figure 25) as two lateral rows rather than spirally arranged rows as occurs in other Australian species of *Unisaccus*. Therefore, I consider *Unisaccus sprenti* as *incertae sedis. Unisaccus sprenti* most likely has a close relation to one of those species from *Valamugil* spp. in the Indo-West Pacific region (see Blasco-Costa, Gibson et al., 2009).

Nomen nudum

Unisaccus corsulai Datta, Manna, & Kundu, 1997

The name is found in an abstract (Datta et al., 1997) for a species from *Rhinomugil corsula* Hamilton, corsula (Mugilidae), that does not differentiate it from other members of the genus.

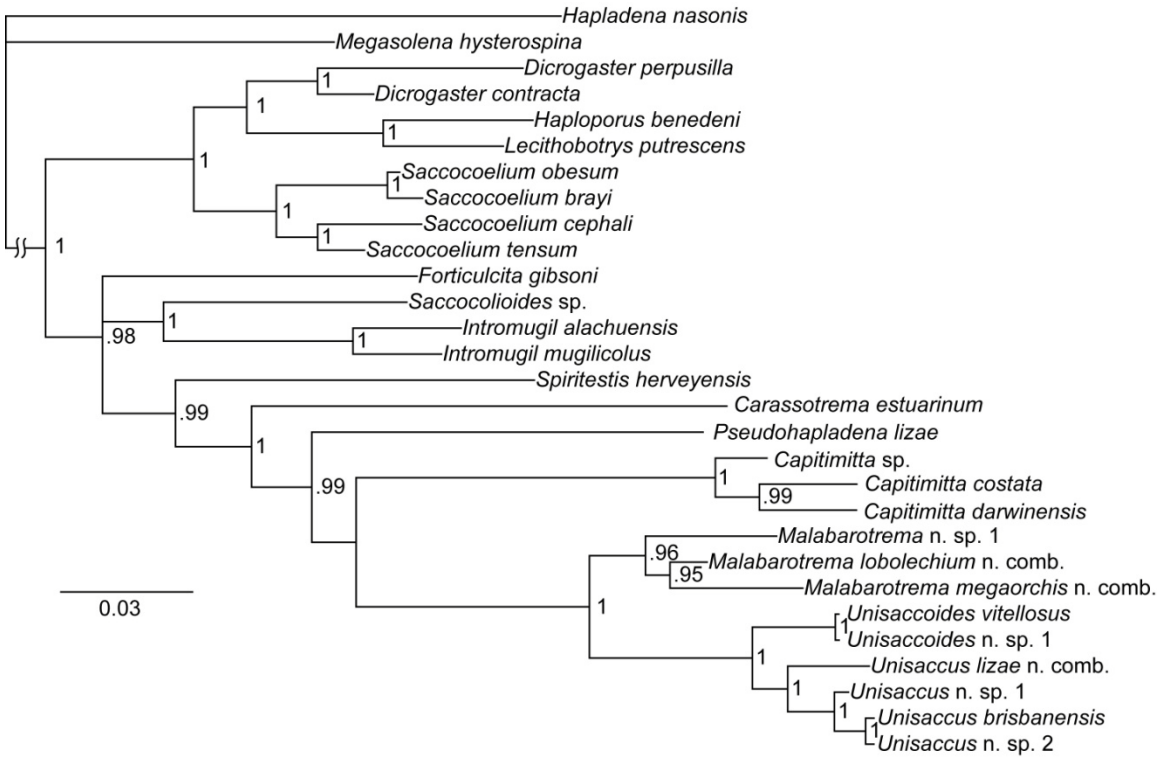


Figure IV.20. Estimated position of species of *Malabarotrema*, *Unisaccus*, and *Unisaccoides* within the Haploporidae Phylogenetic relationships of the Haploporidae using partial 28S rDNA sequences. Posterior probability score is given at the nodes (See Table 1.

Skrjabinolecithum Belous, 1954.

I consider *Skrjabinolecithum* monotypic for *S. spasskii* Belous, 1954. The defining characteristics of *Skrjabinolecithum* are short prepharynx, tapiform vitellarium, hermaphroditic duct without pads, and bifurcating caeca, with the bifurcation anterior to the majority of the hermaphroditic sac; all of these characteristics differentiate the genus from other genera of the Haploporidae.

Molecular data

I compared DNA sequence data from three species of *Malabarotrema*, two species of *Unisaccoides*, and four species of *Unisaccus*. The fragment sequenced encompasses the 3' end of the 18S gene, the ITS region (ITS1 + 5.8S + ITS2), and about

Table IV.1

Sequences used for phylogenetic analysis in this study.

Species	Country	Host	GenBank†
Hapladena nasonis	Australia	*Naso unicornis*	AY222265.1
*Megasolenae hysterospina**	USA	*Lagodon rhomboides*	EP-625
Dicrogaster contracta	Spain	*Liza aurata*	FJ211261.1
Dicrogaster perpusilla	Spain	*Liza ramado*	FJ211238.1
Forticulcita gibsoni	Spain	*Mugil cephalus*	FJ211239.1
Haploporus benedeni	Spain	*Liza ramado*	FJ211237.1
Lecithobotrys putrescens	Spain	*Liza saliens*	FJ211236.1
Saccocoelium brayi	Spain	*Liza saliens*	FJ211234.1
Saccocoelium cephali	Spain	*Mugil cephalus*	FJ211233.1
Saccocoelium obesum	Spain	*Liza ramado*	FJ211259.1
Saccocoelium tensum	Spain	*Liza aurata*	FJ211258.1
Saccocoelioides sp.	Nicaragua	*Poecilidae*	EF032696.1
Capitimitta costata	Australia	*Selenotoca mulifasciata*	KC206497.1
Capitimitta darwinensis	Australia	*Selenotoca multifasciata*	KC206498.1
Capitimitta sp.	Australia	*Selenotoca multifasciata*	KC206499.1
Spiritestis herveyensis	Australia	*Moolgarda seheli*	KC206500.1
*Carassotrema estuarium**	China	*Mugil cephalus*	EP-198
*Pseudohapladena lizae**	China	*Mugil cephalus*	EP-620
Intromugil alachuaensis	USA	*Mugil cephalus*	KC430095.1
Intromugil mugilicolus	USA	*Mugil cephalus*	KC430096.1
*Malabarotrema lobolecthium**	Australia	*Paramugil georgii, Moolgarda seheli Moolgarda perusii*	EP-568
*Malabarotrema megaorchis**	Australia	*Mugil cephalus*	EP-644
Malabarotrema n. sp. 1*	Australia	*Paramugil georgii, Moolgarda seheli*	EP-148
*Unisaccoides vitellosus**	Australia	*Chelon subvirdis*	EP-379
Unisaccoides n. sp. 1*	Australia	*Chelon macrolepis*	EP-077
*Unisaccus brisbanensis**	Australia	*Chelon subvirdis*	EP-376
*Unisaccus lizae**	China	*Mugil cephalus*	EP-640
Unisaccus n. sp. 1*	Australia	*Chelon planiceps*	EP-277
Unisaccus n. sp. 2*	Australia	*Chelon macrolepis*	EP-591

* new sequence data produced for this study

† representative sequence to be deposited in GenBank

1,350 bp of the 5' end of the 28S gene because this region has been shown to be suitable for species differentiation in the haploporids (Blasco-Costa, Balbuena et al., 2009; Blasco-Costa et al., 2010; Pulis & Overstreet, 2013; Pulis et al., 2013). No intraspecific variation was found in cases when sequences were obtained from multiple individuals of each species. The BI analysis of partial 28S rDNA gene sequences (Figure 20) included *Hapladena nasonis* as the outgroup, *Megasolena hysterospina* and an ingroup of 27 non-Megasolenae haploporids. The ingroup of haploporids form a monophyletic clade. The haploporine genera *Dicrogaster*, *Haploporus*, *Lecithobotrys*, and *Saccocoelium* formed a monophyletic clade sister to a polytomy formed by three groups: (1) *Forticulcita gibsoni*, (2) *Saccocolioides* sp. + *Intromugil*, and (3) *Spiritestis*, *Carassotrema*, *Capitimitta*, *Paraunisacoides*, and *Unisaccus*. The three species of *Malabarotrema* form a clade sister to the six species of *Unisaccoides* and *Unisaccus*.

Discussion

The most recent treatments of the Haploporinae (Overstreet & Curran 2005; Blasco-Costa, Gibson, Balbuena, Raga, & Kostadinova 2009; Blasco-Costa, Montero, Gibson, Balbuena, & Kostadinova 2009) all included *Unisaccus* within the Haploporinae. According to my analysis, *Unisaccus* is most closely related to *Unisaccoides*, which were formerly in the genera *Skrjabinolecithum* of the waretrematines. Thus, to make Haploporinae monophyletic, I must (1) reduce the number of subfamilies of Haploporidae to only Megasolenae and Haploporinae, (2) resurrect the Unisaccinae and propose several new subfamilies, or (3) transfer *Unisaccus* to Waretrematinae. Synonymizing Waretrematinae, Chalcinotrematinae, and Forticulcitinae would result in the oversimplification of the relations within the group based on a small sampling of the

recognized species in the haploporidae. Resurrecting the *Unisaccinae* would require the establishment of several new subfamilies according the phylogeny produced here, with *Spiritestis* Nagaty, 1948, *Carassotrema* Park, 1938, *Pseudohapladena* Belous, 1954, and *Capitimitta* Pulis and Overstreet, 2013 each being members of a separate subfamily. That option would reduce the value of subfamilies because presently accepted subfamilies would basically become genera. Transferring species of *Uninsaccus* to the Waretrematinae is the most parsimonious option in my opinion, even though this option reduces the phyletic utility of morphological characters for separating the subfamilies as currently defined because members of *Unisaccus* exhibit two small clusters of vitelline follicles lateral to the ovary and in-utero eggs containing miracidium with developed eye-spots. Regardless, like the species of *Unisaccus* treated here, there are likely other genera and species that are misplaced and will require substantial reclassification of the Haploporidae subfamilies.

Pseudolecithobotrys Blasco-Costa, Gibson, Balbuena, Raga, and Kostadinova, 2009 was proposed to accommodate *Lecithobotrys stomachicola* Machida, 1996, another species that can exhibit a single caecum (Machida, 1996, Blasco-Costa, Gibson et al., 2009). In contrast to the species of *Malabarotrema* and *Unisaccus* treated here and by others (Martin, 1973b, 1973c, Liu, 2002), I have not observed such dramatic variation in caecal configuration in the species I consider *Malabarotrema, Unisaccoides,* or *Unisaccus*. I agree with Blasco-Costa, Gibson, et al. (2009) that *Pseudolecithobotrys* is a valid genus and is differenciated from *Unisaccus* by (1) eye-spot pigment absent, (2) hermaphroditic sac about about three time as long as wide, arcuate, (3) female duct about, (4) hermaphroditic duct lined intensely staining cells, and (5) eggs thick-shelled. The

position of *Pseudolecithobotrys* within the haploporids is not certain. The variable caeca/ caecum perhaps could point to a position basal to the *Malabarotrema, Unisaccoides,* and *Unisaccus* clade with caecum shape having been fixed in the *Malabarotrema* and *Unisaccus* species. Alternatively, several morphological features provide stronger evidence for a closer relation of *Pseudolecithobotrys* of the Haploporinae; short prepharynx, long esophagus, long narrow hermaphroditic sac, and absence of eye-spot pigment in adults, but this is probably an oversimplification of morphological characters.

Table IV.2

Number of variable sites based on pairwise sequence comparison of the ITS1 (c. 380 pb) region (above diagonal) and the 5.8S (c. 157 bp) gene (below diagonal) of nuclear ribosomal DNA among 3 species of Malabarotrema, 2 species of Unisaccoides, and 4species of Unisaccus. Malabarotrema lobolecthium not included in ITS1 comparison due to long indels.

	Malabarotrema lobolecthium	*Malabarotrema. megaorchis*	*Malabarotrema n. sp. 1.*	*Unisaccoides vittelosus*	*Unisaccoides n. sp. 1*	*Unisaccus brisbanensis*	*Unisaccus lizae*	*Unisaccus* n. sp. 1	*Unisaccus* n. sp. 2
M. megaorchis	2	-	108	137	137	132	151	130	132
M. n. sp. 1	0	2	-	119	119	142	160	152	142
Unisaccoides vittelosus	0	2	0	-	2	60	108	58	60
Unisaccoides n. sp. 1	1	3	0	0	-	60	108	58	60
Unisaccus brisbanensis	1	3	1	1	1	-	81	22	0
Unisaccus lizae	0	2	1	0	1	1	-	89	81
Unisaccus n. sp. 1	0	2	0	1	1	0	0	-	22
Unisaccus n. sp. 2	0	2	0	1	1	0	0	0	-

Table IV.3

Number of variable sites based on pairwise sequence comparison of the ITS2 (c. 280) region (above diagonal) and about 1,360 bases at the 5' end of the 28S gene (below diagonal) of nuclear ribosomal DNA among 3 species of Malabarotrema , 2 species of Unisaccoides, and 4 species of Unisaccus.

	Malabarotrema lobolecithum	*Malabarotrema megaorchis*	*Malabarotrema n. sp. 1.*	*Unisaccoides vittelosus*	*Unisaccoides n. sp. 1*	*Unisaccus brisbanensis*	*Unisaccus lizae*	*Unisaccus* n. sp. 1	*Unisaccus* n. sp. 2
M. lobolecthium	-	21	25	36	34	43	37	45	44
M. megaorchis	34	-	25	36	34	42	36	42	42
M. n. sp. 1	37	53	-	40	38	51	47	50	50
Unisaccoides vittelosus	61	70	80	-	2	21	21	22	20
Unisaccoides. n. sp. 1	61	70	80	0	-	19	19	20	18
Unisaccus brisbanensis	62	71	78	36	36	-	20	19	19
Unisaccus lizae	66	75	82	40	40	34	-	19	19
Unisaccus n. sp. 1	62	68	74	34	34	10	62	-	8
Unisaccus n. sp. 2	63	72	79	36	34	2	34	10	-

Unicoelium prochilodorum Thatcher and Dossman, 1975 bears a striking resemblance to species of *Unisaccus*, but my proposing a synonymization at this time would be based on characters that do not appear to be of systematic importance. The caecum, Y-shaped excretory vesicle, in-utero eggs containing miracidium with eye-spots, and two clumps of vitelline follicles all show great similarity with species of *Unisaccus*, a quarter or less the length of the hermaphroditic sac (referred to as metraterm by Blasco-Costa, Gibson, et al. 2009), but other than species with a caecum, other members of the Haploporinae (*Saccocoelium*, *Haploporus*) and Chalcinotrematinae (*Saccocoelioides*,

Paralecithobotries) share these features as well. I do not consider the non-mugilid host to be of much significance as other genera of species primarily infecting mugilids have representatives hosted by non-mugilids, but, based on restriction of other species to the Indo Pacific Region, I do consider a freshwater South American host to be highly unlikely for a species of *Unisaccus*. Formerly, many species and genera within the haploporids have been misallocated based on features that are not of phyletic significance, such as *Intromugil* within the Waretrematinae (Figure 20), *Forticulcita* Overstreet, 1981 within the Haploporinae (Blasco-Costa, Balbuena, et al., 2009), *Spiritestis* Nagaty, 1948 and *Capitimitta* spp. Pulis & Overstreet, 2013 as synonyms of *Waretrema* Srivastava, 1937. I consider the similarity of *Unicoelium* to *Unisaccus* to be a product of convergent evolution and await molecular data to test this hypothesis. Extent of the uterus and position of the hermaphroditic sac and vitellarium of *Unicoelium* support its relation with Chalcinotrematinae and radiation of the freshwater forms in South America. The presence of a developed miracidium with pigmented eye-spots in in-utero eggs have long been one of the characters used to make differentiations among groups within the haploporids (see Overstreet & Curran, 2005). My proposal to remove *Unisaccus* species from the Haploporinae and incorporate them into Waretrematinae changes the characters that can be used to seperate the subfamilies and casts doubt on the current organization of the family. Baysian Inference analysis suggests that *Intromugil* Overstreet and Curran, 2005 is more closely related to *Saccocoelioides* sp. of the chalcinotrematines than to waretrematines as proposed by Shireman (1964) and Overstreet and Curran (2005). I advocate the transfer of *Intromugil* to Chacinotrematinae. Apparently, there has been considerable convergence in characters of the haploporids; at

Table IV.4

COI sequence data for some mullet host of species of Malabarotrema, Unisaccoides, and Unisaccus (Fishbase accessed 12/2013)

FAO identification	FishBase current name	Host #	GenBank similarity	Durand et al. proposed classification
Liza macrolepis	*Chelon macrolepis* (Smith, 1846)	460	99% JQ060418	*Planiliza macrolepis* (Smith, 1846)
Valamugil georgii	*Paramugil georgii* (Ogilby, 1897)	462	85% JQ060445	†
Liza planiceps	*Chelon planiceps* (Valenciennes, 1836)	497	99% JQ060429	*Planiliza* sp. F
Valamugil perusii	*Moolgarda perusii* (Valenciennes, 1836)	520	100% JQ060496	*Osteomugi*l sp. A
Valamugil perusii	*Moolgarda perusii* (Valenciennes, 1836)	521	99% JQ060496	*Osteomugil* sp. A
Liza planiceps	*Chelon planiceps* (Valenciennes, 1836)	525	97% JQ060488	*Planiliza**
Liza planiceps	*Chelon planiceps* (Valenciennes, 1836)	525	99% JQ060429	*Planiliza* sp. F
Valamugil seheli	*Moolgarda seheli* (Forsskål, 1775)	569	100% JQ060516	*Crenimugil* sp. C
Valamugil seheli	*Moolgarda seheli* (Forsskål, 1775)	569	100% JQ060516	*Crenimugil* sp. C
Liza subviridis	*Chelon subviridis* (Valenciennes, 1836)	601	98% JQ060432	*Planiliza subviridis* (Valenciennes, 1836)
Liza subviridis	*Chelon subviridis* (Valenciennes, 1836)	601	98% JQ060432	*Planiliza subviridis* (Valenciennes, 1836)

† GenBank nucleotide blast search most similar sequence 85%. I believe this to be the first published COI sequence obtained from *Paramugil georgii* (Ogilby, 1897).

* In the *Planiliza* clade, 97% similar to *Planizia* sp. C

this time more molecular data is needed to better understand diversification of the family and to determine which characters are of systematic value.

I have removed from *Skrjabinolecithum* members of two genera with a caecum rather than two caeca. The monotypic *Skrjabinolecithum spasski* Belous, 1954 possesses two sac-like or tubular caeca, a tapeiform vitellarium, caecal bifurcation in the forebody, and a hermaphroditic sac almost entirely anterior to the ventral sucker. At least some of these characters are of generic significance and preclude a natural grouping of *S. spasski* with *Unisaccoides* or *Malabarotrema* species.

To accurately describe and understand the diversity of the haploporids, their habitats and definitive hosts should be accurately known. Unfortunately, there exists uncertainty in the taxonomy of mullets. When I collected some of the fish used in this study, I saved a tissue sample to confirm the identity of the fish host. With the recent sequencing work of Durand, Shen et al. (2012), I now have access to comparable sequences with those fish I collected. For the most part, FAO keys (Harrison & Senou, 1997) allow accurate identification of the mullet species of Australia, based on identification assistance and confirmation from R. Williams NTM and Jeff Johnson QM. Until mullet taxonomy is better understood, I treat all host identifications with caution, especially when comparing what has been thought to be the same host species across large geographic distances. With the somewhat unexpected diversity of the mullets, more emphasis in the future should be put towards understanding the patterns of diversification of haploporid lineages with their associated mullet lineages.

Key to genera and species of Waretrematinae possessing undivided caecum.

1a. Body elongate; pharynx at anterior margin of ventral sucker or further posterior; excretory vesicle terminating at about mid hindbody; vitellarium consisting of large areas of occupying most of hindbody ..... *Malabarotrema* Zhukov, 1972

b. Body fusiform; prepharynx terminating prior to ventral sucker; vitellelarium reduced to clumps lateral to ovary or small areas of follicles and sparse in hindbody .....2.

2a. Mature in-utero eggs containing underdeveloped miracidium without eye-spots .... *Unisaccoides* Martin, 1973

b. Mature in-utero eggs containing miracidium with eye-spots ... *Unisaccus* Martin, 1973

Malabarotrema

1a. Area between genital pore and oral sucker with only a few irregularly spaced spines. 2

b. Area between genital pore and oral sucker on ventral surface covered in spines of the same size and pattern or other tegument spines 3

2a. 3-4 rows of enlarged spines dorsal to the oral sucker.. ....*Malabarotrema lobolecthium* (Martin, 1973) n. comb.

b. Spines dorsal to oral sucker not enlarged compared to body spines ....*Malabarotrema indica* Zhukov, 1972

3a. Body width ≥ 27% of BL; egg length 58-71..... .....*Malabarotrema megaorchis* (Lui & Yang, 2002) n. comb.

b. Body width ≤ 27%; egg length 68-88.....*Malabarotrema* n. sp. 1

Unisaccoides

1a. Pharynx shorter than 0.9 length of prepharynx....*Unisaccoides vitellosus* Martin, 1973

b. Pharynx longer than 1.0 length of prepharynx ..... *Unisaccoides* n. sp. 1

Unisaccus

1a. Ventral sucker noticeably smaller than oral sucker; sucker ratio always 1:<0.7 .....2.

b. Ventral sucker smaller or larger than oral sucker; ratio 1:>0.7 .....3.

2a. Ovary and testis separated; mature eggs about same size as ventral sucker..... ..... *Unisaccus gupttai* (Ahmad, 1987) Overstreet & Curran, 2005

b. Ovary and testis nearly contiguous; mature eggs larger than ventral sucker..... ..... *Unisaccus martini* Ahmad, 1986

3a. Ovary larger than testis .....

..... *Unisaccus overstreeti* (Ahmad, 1987) Overstreet & Curran, 2005

b. Testis larger than Ovary.....4.

4a. Eggs significantly larger than ventral and oral suckers .....

.....*Unisaccus mugilis* (Rekharani & Madhavi, 1985) Blasco-Costa, Montero, Gibson, Balbuena, Raga, & Kostadinova, 2009

b. Eggs not significantly larger (usually smaller) than ventral and oral suckers.....5.

5a. Vitellarium composed of four groups of lobes in H-pattern (including vitelline duct), lateral anterior part near into ventral sucker region, posterior part into testicular region .....*Unisaccus lizae* Liu, 2002

b. Vitellarium composed of 2 groups of lobes, largely confined to region between ventral sucker posterior and testicular anterior margin.....6.

6a. Mature eggs shorter than pharynx.....7.

b. Mature eggs longer than pharynx .....8.

7a. Prepharynx shorter than pharynx ..... *Unisaccus* n. sp 1.

b. Prepharynx longer than pharynx..... .*Unisaccus spinosus* Martin, 1973

8a. Hermaphroditic sac terminating at or anterior to posterior margin of ventral sucker; hosted by *M. cephalus* and *L. subvirdis*, in eastern Australia.....

.....*Unisaccus brisbanensis* Martin, 1973

b. Hermaphroditic sac terminating at or posterior to posterior ventral sucker margin; hosted by *L. macrolepis*, in western Australia ..... *Unisaccus* n. sp 2

CHAPTER V

A NEW SPECIES OF *CARASSOTREMA* (TREMATODA; HAPLOPORIDAE) AND NOTES ON SOME OTHER MEMBERS OF THE GENUS

Abstract

Carassotrema Park, 1938 is revised. A new species is described from the Australian clupeid *Nematalosa come*. *Carassotrema estuarinum* Tang and Lin, 1979, *C. ginezinskajae* Kulakova and Ha Ky, 1976, *C. heterorchis* Wang and Pan, 1984, *C. kui* Tang and Lin, 1979, *C. pterorchis*, Wang, 1973, and three unidentified species are characterized morphologically and molecularly based on new material. *Carassotrema lizae* Al-Daraji, 1999 is considered a synonym of *C. bengalense* Rakharani and Madhavi, 1985. Phylogenetic hypotheses based on partial ITS region and partial 28S gene along with those reported genes from other haploporids show (1) species of *Carassotrema* used herein form a monophyletic clade, (2) species of *Carassotrema* are well supported within the Waretrematinae Srivastava, 1937, (3) species that were formerly allocated to *Platydidymus* Overstreet and Curran, 2005 do not form a monophyletic clade separate from species Overstreet and Curran (2005) considered within *Carassotrema*, and (4) species from cyprinid hosts do not form a monophyletic clade when species from mugilids and clupeids are included. *Platydidimus* is accepted as monophyletic for *Platydidimus flecterotestis* (Zhukov, 1971). Discrepancies in authority dates of species are disscussed when necessary. The following species are accepted in the genus *Carassotrema*: *C. koreanum* Park, 1938, *C. bengalense* Rakharani and Madhavi, 1985, *C. clupanodonae* Liu, 2003, *C. estuariunum*, *C. ginezinskajae*, *C. heterorchis*, *C. kui*, *C.*

lemellorchis Wang, 1973, *C. megapharyngus* Wang, 1973, *C. philippinensis* Machida, 1996, *C. pterorchis, C. schistorchis* Wang and Pan, 1984, and the new species.

Introduction

Carassotrema Park, 1938 was established with *C. koreanum* Park, 1938 as the type-species for specimens obtained from *Carassius auratus* (Linnaeus) (Cyprinidae) (Park, 1938). Without formally placing it in a family, Park made comparisons of *C. koreanum* with Allocreadidae Looss, 1902 and Monorchidae Odhner, 1911 and differentiated from *Deradena ovalis* Linton, 1910, now *Hapladena ovalis* (Linton, 1910) Manter, 1947, a haploporid. The number of species and geographic distribution of *Carassotrema* has been reviewed several times (Nasir & Gomez, 1976; Tang & Lin, 1979; Rekharani & Madhavi, 1985; Moravec & Sey, 1989; Kohn et al., 1999) The most recent treatments of the genus have been conflicting. Overstreet and Curran (2005) erected *Platydidymus* Overstreet and Curran, 2005 for the preoccupied *Haplotrema* Zhukov, 1971 and added four other species originally described in *Carassotrema* that possess wider than long testis located near the posterior extremity of the body, including *C. philippense* Machida, 1996, C. *megaphyryngus* Wang, 1973 (including tenative syns. *C. ginezinskajae* Kulakova and Hy Ka, 1976 and *C. heterosacca* Pan, 1965), *C. heterorchis* Wang and Pan 1984, and *C. wui* Tang and Lin, 1963. Conversely, Yu et al. (2005) synonomized the preoccupied *Haplotrema* with *Carassotrema* and included 14 species in the genus, including *Intromugil mugilicolus* (Shireman, 1964) Overstreet and Curran, 2005 and *Culuwiya tilapiae* (Nasir & Gomez, 1976) Overstreet and Curran, 2005. I agree with Overstreet and Curran (2005) in their placement of *Culuwiya tilapiae* and

Intromugil mugilicolus outside of *Carassotrema*, but consider the species they placed in *Platydidymus*, other than *P. flecterotestis*, as *Carassotrema* spp.

Only one species of *Carassotrema* has been described from a clupeid, *C. clupanodonae* Liu, 2003 (Liu, 2003); usual hosts are Mugilidae (3 spp.) and Cyprindae (7 spp.). No species of *Carassotrema* has been reported from the Southern Hemisphere. I collected an undescribed species of *Carassotrema* from *Nematalosa come* (Richardson) (Clupeidae) off Australia. Herein, I describe the new species and provide morphological and molecular data from eight species of *Carassotrema* collected from China. Due to the paucity of type specimens, differential diagnoses rely heavily on original descriptions and subsequent publications by the original authors.

Materials and Methods

Chinese specimens were obtained from hosts purchased live at fish markets, and those hosts were assumed to be from the nearby water bodies. Australian specimens were recovered from fish collected by cast net. No prevalence data is provided due to lack of knowledge of exact capture location of the fishes. Further, most parasite collections were from pooled gut wash of several individuals of the same species. Specific fish names follow those given by FishBase (Froese & Pauly, 2013), with the exception of subspecies designations. Live worms were rinsed and cleaned in a container with saline and examined briefly. Following gross examination, most of the saline was removed from the container, and the worms were killed by pouring hot steaming water (not boiling) over them. This procedure was followed by fixation in 70% ethanol. When the number of specimens of a species allowed, a few other specimens were placed at room temperature directly into 95% ethanol for molecular analysis, and a few others were heat-killed while

under coverslip pressure for the critical examination of ducts and comparision with other descriptions. Worms were stained in aqueous alum carmine, Mayer's haematoxylin or Van Cleave's haematoxylin, dehydrated in a graded ethanol series, cleared in clove oil (carmine and Van Cleave's) or methyl salicylate (Mayer's), and mounted permanently in Damar gum. Measurements were taken using a Leica compound microscope equipped with differential interference contrast (DIC) and ProgRes® CapturePro camera (Version 2.8 Jenoptic, Jena, Germany) and software. All measurements are in micrometres unless otherwise noted. Museum abbreviations are as follows: ISCAS, Helminthological Collection of the Institute of Parasitology, Branišovská, Czech Republic; NSMT, National Science Museum, Tokyo; QM, Queensland Museum, Brisbane, Australia; RAS, Russian Academy of Sciences, Parasitic Worms Division, St. Petersburg, Russia; and USNPC, US National Parasite Collection, Beltsville, Maryland, USA.

Museum material examined consisted of specimens from the ISCAS labelled as *Carassotrema koreanum* D 214/1 (2 slides), D 214/2 (2 slides), D 214/3; from NSMT *Carassotrema philipinensis* NSMT-Pl 3939 1/2, 3939 2/2, 3925 2/3, 3925 3/3 (multiple specimens on each slide); from RAS Parasitic Worms Division *Carassotrema ginezinskajae*, No. 6-168 (5), pictures taken by Dr. Irina Podvyaznaya of type series and *Platydidymus flecterotestis*, No. 1.7.58 several specimens on one slide. Other attempts to borrow vouchers and types were unsuccessful. The types of *C. schistorchis*, *C. heterorchis*, and vouchers associated with Wang and Pan (1984) could not be located (pers. comm. G.T. Wang, Institute of Hydrobiology).

Genomic DNA was isolated using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the instructions provided. DNA fragments *c*. 2,500

base pairs (bp) long, comprising the 3' end of the 18S nuclear rDNA gene, internal transcribed spacer region (including ITS1 + 5.8S + ITS2), and the 5' end of the 28S 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 primers ITSF (5' - CGCCCGTCGCTACTACCGATTG-3') or LSU5 (5'-TAGGTCGACCCGCTGAAYTTAAGCA-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'). Internal reverse primers were 300R (5'-CAACTTTCCCTCACGGTACTTG-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., Foster City, California, USA), ethanol-precipitated and run on an ABI 3130 Genetic Analyzer™. Contiguous sequences were assembled using Sequencher™ 5.10 (GeneCodes Corp., Ann Arbor, Michigan, USA, Version) and submitted to GenBank.

Newly obtained sequences and sequences of other haploporids were used in phylogenetic analyses. Sequences obtained from GenBank and used in the present analysis are as follows: *Capitimitta costata* Pulis & Overstreet, 2013 KC206497; *Capitimitta darwinensis* Pulis & Overstreet, 2013 KC206498; *Capitimitta* sp. of Pulis and Overstreet, 2013 KC206497; *Dicrogaster contracta* Looss, 1902 FJ211261; *Dicrogaster*

perpusilla Looss, 1902 FJ211238; *Forticulcita gibsoni* Blasco-Costa, Montero, Gibson, Balbuena, and Kostadinova, 2009 FJ211239; *Hapladena nasonis* Yamaguti, 1970 AY222265; *Haploporus benedeni* Looss, 1902 FJ211237; *Intromugil mugilicolus* (Shireman, 1964) KC430096; *Intromugil alachuaensis* Pulis et al., 2013 KC430095; *Lecithobotrys putrescens* Looss, 1902 FJ211236; *Megasolena hysterospina* (Manter, 1931) EP-625; *Malabarotrema* n. sp. 1 Pulis, Chapter IV. EP-148; *Malabarotrema lobolecithium* Martin, 1973 EP-568; *Megbarotrema megaorchis* (Lui & Yang, 2002) EP-644; *Pseudohapladena lizae*, Liu and Yang, 2002 EP-620; *Saccocoelioides* sp. EF032696; *Saccocoelium brayi* Blasco-Costa, Montero, Gibson, Balbuena, Raga, and Kostadinova, 2009 FJ211234; *Saccocoelium obesum* Looss, 1902 FJ211259; *Saccocoelium tensum* Looss, 1902 FJ211258; *Saccocoelium cephali* Blasco-Costa et al., 2009 FJ211233; *Spiritestis herveyensis* Pulis and Overstreet, 2013 KC2006500; *Unisaccoides vittelosum* (Martin, 1973) EP-379; *Unisaccus brisbanensis* Martin, 1973 EP-376; and *Unisaccus lizae* (Liu, 2002) EP-640.

Previously published 28S rDNA gene sequences and new sequences were used for comparison (see Table 1). The 28S alignment of 34 haploporid species was generated in ClustalX 2.1, masked with GBlocks using default settings with gaps set to "some" (Talavera & Castresana, 2007), verified in Bioedit, and trimmed to the shortest sequence on each end. The Baysian Inference (BI) analysis of partial 28S gene was run using a dataset consisting of 33 Haploporidae taxa and *Hapladena nasonis* (Megasolenae) as the out group. The alignment included a total of 1,185 sites, including gaps. Positions excluded were generated using GBlocks software (Castresana, 2000; Talavera & Castresana, 2007), set to *gaps = some* with all other options left as default. BI analysis of

the ITS region (including partial ITS1, 5.8S, and ITS 2) the 5' end of the ITS 1 trimmed and ~380 of the 3' were analyzed. The resulting ITS alignment included 920 sites. The best nucleotide substitution model was estimated with jModeltest Version 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) as general time reversible with estimates of invariant sites and gamma-distributed among site-rate variation (GTR + I + Γ) (partial 28S) and general time reversible gamma-distributed among site-rate variation (GTR + Γ) (Partial ITS region). Phylogenetic analysis of the data was performed using Bayesian Inference (BI) with MrBayes 3.1.2 software (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) and *H. nasonis* or *S. herveyensis* as the outgroup (28S and ITS data sets, respectively). For 28S tree the model parameters used were 28S tree nst = *6*, rates=*invgamma*, ngen = *1,000,000*, and samplefreq = *100*. For the ITS tree the model parameters used were nst = *6*, rates = *gamma*, ngen = *1,000,000*, and samplefreq = *100*. By 250,000 generations the split deviations had stabilized for both data sets and the first 25% of samples were discarded (*sump burnin*) and nodal support was estimated by posterior probabilities (*sumt burnin*) (Huelsenbeck et al., 2001), with all other settings left as default.

Carassotrema Park, 1938

Diagnosis: Body fusiform to pyriform. Eye-spot pigment diffuse. Oral sucker globular, terminal to subterminal, mouth opening anteriorly to anteroventrally. Prepharynx indistinct to longer than pharynx. Pharynx well-developed, larger or smaller than suckers. Ventral sucker usually larger than oral sucker. Oesophagus distinct. Caeca long, cylindrical terminating in testicular space or further posterior. Testis shape variable, U, Y, V, irregular, or elongate, in hindbody. Hermaphroditic sac ellipsoidal to elongate,

hermaphroditic duct often longer than half the length of sac. Internal seminal vesicle usually half or more length of hermaphroditic sac. External seminal vesicle sac-like, usually as large or larger than internal seminal vesicle. Ovary often bean-shaped, directed ventrally, usually contiguous with testis, often between lobes of testis. Seminal receptacle canicular with or without swelling proximally. Mehlis' gland near distal end of ovary. Uterus confined to area between testis and hermaphroditic sac. Eggs in utero few, relatively large, shell yellow, lacking developed miracidium with eye-spots. Miracidium assumed to develop eye-spots after deposition in all species. Excretory vesicle Y-shaped; pore terminal. In marine and freshwater fishes (Mugilidae, Clupeididae, Cyprinidae, Bagridae, Channidae), Indo West Pacific Region.

Carassotrema koreanum Park, 1938

Syns Asymphylodora pavlovskajae Ha Ky, 1969 *nomen nudum*

Material studied: *Saurogobio dabryi* Bleeker, 1871 D214_2a, D214, D214 _2b, D214_3; *Hemiculter leucisculus* (Basilewsky).

Records

References: 1. Park (1938) 2. Tang and Lin (1963) 3. Wang (1973) 4. Yamaguti (1971) 5. Kulakova and Ha Ky (1976), 6. Tang and Lin (1979) 7. Wang et al., (1983) 8. Wang and Pan, (1984), 9. Bykhovskaya-Pavlovskaya and Kulakova (1987), 10. Moravec and Sey (1989) 11. Wang (1991) 12. Wang and Zhou (1993).

Descriptions: 1; 3; 5; 6; 8; 9; 10; 11; 12.

Distribution: China: Fujian (2, 6, 8), Guangdong (8), Hubei (3, 8), Hunan (8), Jiangsu (7, 8), Jiangxi (12), Zhejiang (8, 11). Japan: not specified (4). Korea: near Seoul (1). Russia: Amur Basin (9). Vietnam: Bac-kan (5), Lao-cai (5), near Hanoi (10).

Definitive Hosts: *Acheilognathus taenianalis* (Günther) (3, 11, 12), no common name (Cyprinidae); *Carassius auratus* (Linnaeus) (type host) (1, 2, 3, 4, 6 [experimental and wild], 7, 8, 11, 12), goldfish (Cyprinidae); *Channa argus* (Cantor) (7), snakehead (Channidae); *Chanodichthys dabryi* (Bleeker) (8, 11), humpback (Cyprinidae); *Chanodichthys erythropterus* (Basilewsky), predatory carp (Cyprinidae); / *Culter alburnus* Basilewsky* (8, 11, 12), no common name (Cyprinidae); *Chanodichthys mongolicus* (Basilewsky) (8, 11, 12), Mongolian redfin (Cyprinidae); *Cirrhinus molitorella* (Valenciennes) (5), mud carp (Cyprinidae); *Ctenopharyngodon idella* (Valenciennes) (3, 8, 11, 12), grass carp (Cyprinidae); *Cyprinis carpio* Linnaeus (3, 4, 6, 7, 8, 11, 12), common carp (Cyprinidae); *Hemiculter leucisculus* (Basilewsky) (8, 10, 11, 12), sharpbelly (Cyprinidae); *Hypophthalmichthys molitrix* (Valnciennes) (3, 9, 12), silver carp (Cyprinidae); *Hypophthalmichthys nobilis* (Richarson) (3, 12), bighead carp (Cyprinidae); *Megalobrama amblycephala* Yih (8, 11), Wuchang bream (Cyprinidae); *Megalobrama terminalis* (Richardson) (11), Black Amur bream (Cyprinidae); *Mylopharyngodon piceus* Richardson (8, 11, 12), black carp (Cyprinidae); *Opsariichthys bidens* (Günther) (6, 8, 11, 12), no common name (Cyprinidae); *Parabramis pekinensis* (Basilewsky) (11), White Amur bream (Cyprinidae); *Squaliobarbus curriculus* (Richardson) (3, 8, 11, 12), barbel chub (Cyprinidae); and *Saurogobio dabryi* Bleeker (10) Chinese lizard gudgeon (Cyprinidae).

* Kottelat (2006) noted that from approximately 1964 until 2001, the synonymies of *Culter alburnus* and *Chanodichthys erythropterus* have been inverted in the Chinese and Southeast Asian literature. Several articles listed two synonyms for *C. erythropterus* (Froese & Pauly, 2013) as definitive host. In light of this, I use *Chanodichthys*

erythropterus (Basilewsky, 1855)/*Culter alburns* Basilewsky, 1855 for the host when either has been listed throughout this paper.

Intermediate host: *Stenothyra glabra* (Adams) (6)

Remarks

Characters useful for the differentiation of *C. koreanum* from congeners include distinct to long prepharynx, oesophagus terminating near posterior margin of ventral sucker, caeca terminating near posterior end of testis, triangular to weakly Y-shaped testis, hermaphroditic sac terminating near posterior margin of ventral sucker, and vitellarium commencing near the level of mid-ventral sucker.

Since its original description, this species has been widely reported, both in definitive hosts and geographic distribution. Due to the unavailability of type and corresponding voucher specimens I have elected to accept most reports as listed by their authors when I cannot definitively exclude the reported specimens as *C. koreanum*. For example, after examining the specimens of Moravec and Sey (1989), I conclude that the specimens from *Hemiculter leucisculus* are *C. ginezinskajae*. Those from *Squaliobarbus curriculus* and *Saurogobio dabryi* are in poor condition; therefore, I cannot exclude them from *C. koreanum* as identified by the authors that collected these specimens.

Carassotrema bengalense Rekharani and Madhavi, 1985

Syns Carassotrema lizae Al-Daraji, 1999.

Records

References: 1. Rekharani and Madhavi (1985) 2. Shameem and Madhavi (1991), 3. Al-Daraji (1999). 4. Hassanine and Ahmed (2002)

Descriptions: 1; 2; 3; 4.

Definitive Hosts: *Chelon macrolepis* (Smith) (3) largescale mullet (Mugilidae), *Moolgarda cunnesius* (Valenciennes) (2) longarm mullet (Mugilidae), and *Mugil cephalus* Linnaeus, Type host (1, 2 (natural and experimental) 4). flathead grey mullet (Mugilidae).

Intermediate host: *Stenothyra blanfordiana* Nevill (2), no common name (Gastropoda).

*Distribution***:** India: Chilka Lake (2), Visakhapatnam (1); Iraq: Khor Abdullah (3)

Remarks

Carassotrema bengalense can be differentiated from congeners by a bottle-shaped body, pharynx smaller than suckers, noticeable prepharynx, caeca terminating in the posterior half of testicular space, elongate testis occupying about 70% of the hindbody, long tube leading to internal seminal vesicle proper, genital pore noticeably anterior to ventral sucker, ovary lateral to testis, and vitellarium commencing at anterior margin of ventral sucker.

I consider *C. lizae* Al-Daraji, 1999 to be a synonym of *C. bengalense*. The description of *C. lizae* has considerable problems (i.e., testis reported as 220-356 but shown in Figure 2a by Al-Daraji (1999) as >500, oesophagus reported as 117-132 long but shown in Figure 2a by Al-Daraji (1999) as >150 (Al-Daraji, 1999)). There appears to be a typographical error in the differential diagnosis that confuses the differentiation of *C. lizae* from *C. bengalense* and *I. mugilicous*, but the main justification for proposing a new species was the difference in the sucker ratio between *C. lizae* and *C. bengalense.* Specimens of both Rekharani and Madhavi (1985) and Al-Daraji (1999) were fixed in AFA under slight coverslip pressure; therefore, the amount of pressure may have changed

the sucker ratios. While *C. lizae* may prove to be a valid species, redescription and collection of new material is needed. Hassanine and Ahmed (2002) were unaware of the description by Al-Daraji (1999) or did not consider *C. lizae* a valid species, and they recorded *C. bengalense* from the Northern Red Sea. From the figured specimen, it appears to have been killed under less coverslip pressure than those used for other descriptions and, therefore, conforms to those descriptions (Rekharani & Madhavi, 1985; Shameem & Madhavi, 1991; Al-Daraji, 1999).

Carassotrema bengalense possesses an external seminal vesicle that has a long tubular portion that almost crosses dorsally the length of the ventral sucker before widening to become the sac-like structure more typical of other members of the genus. The vitellarium is composed of numerous small follicles (compared to other members of the genus) beginning at the anterior margin of the ventral sucker. *Carassotrema bengalense* may not be congeneric with the other species of *Carassotrema*.

Carassotrema estuarinum Tang and Lin, 1979

Records

References: 1. Tang and Lin (1979) 2. Present study.

Descriptions: 1; 2.

Definitive Hosts: *Mugil cephalus* Linnaeus, flathead grey mullet (Mugilidae) (1, 2).

Distribution: China: Fujian (1), Guangdong (2).

Material examined: Two mounted specimens USNPC TBA and one specimen DNA extracted, from *Mugil cephalus*, purchased at a live fish market Da Ya Bay, (22° 42' N, 114° 32'E).

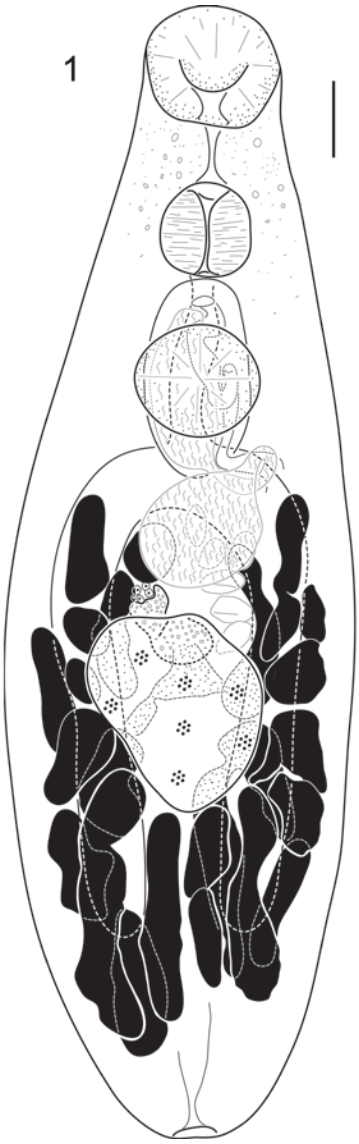


Figure V.1. Carassotrema estuarinum 1. ventral wholemount ex. *Mugil cephalus*; *Scale-bar* 100µm.

Supplementary Data: Table 1, Figure 1

Remarks

C. estuarinum can be differentiated from congeners by body width about 1/3 of BL, suckers small relative to body size, caeca occupying about 50% of BL terminating posterior to testis, testis without deep recess centrally or deep lateral indents, but irregular in outline, post-testicular space greater than about 25% of body length, genital pore

noticeably anterior to ventral sucker, and posterior to pharyngeal region, and vitellarium commencing posterior to ventral sucker.

The description of *C. estuarinum* (Tang & Lin, 1979) states the testis is without lobes or indents, but their Figure 1 shows a shallow recess on the anterior margin of the testis making the testis weakly Y-shaped (Tang & Lin, 1979). My specimens also are weakly Y-shaped to irregular in shape.

Carassotrema clupanodonae Liu, 2003

Records

References: 1. Liu (2003)

Descriptions: 1.

Definitive Hosts: *Konosirus punctatus* (Temminck & Schlegel), dotted gizzard shad (Clupeidae) type host. The species summary designates *Mugil engeli* (Bleeker) as the type host, but all other information in the paper indicates the type host is *K. punctatus* (reported as *Clupanodon punctatus*). I regard the listing of *M. engeli* as unintended.

Distribution: China; Fujian.

Remarks

Carassotrema clupanodonae can be differentiated from congeners by ventral sucker larger than oral sucker, distinct prepharynx, vitellarium commencing near mid-level of ventral sucker, oesophagus terminating near mid-level of ventral sucker, testis elongate and entire, ovary lateral to testis, genital pore between pharyngeal and ventral sucker levels, and hermaphroditic sac reaching only just beyond anterior margin of ventral sucker.

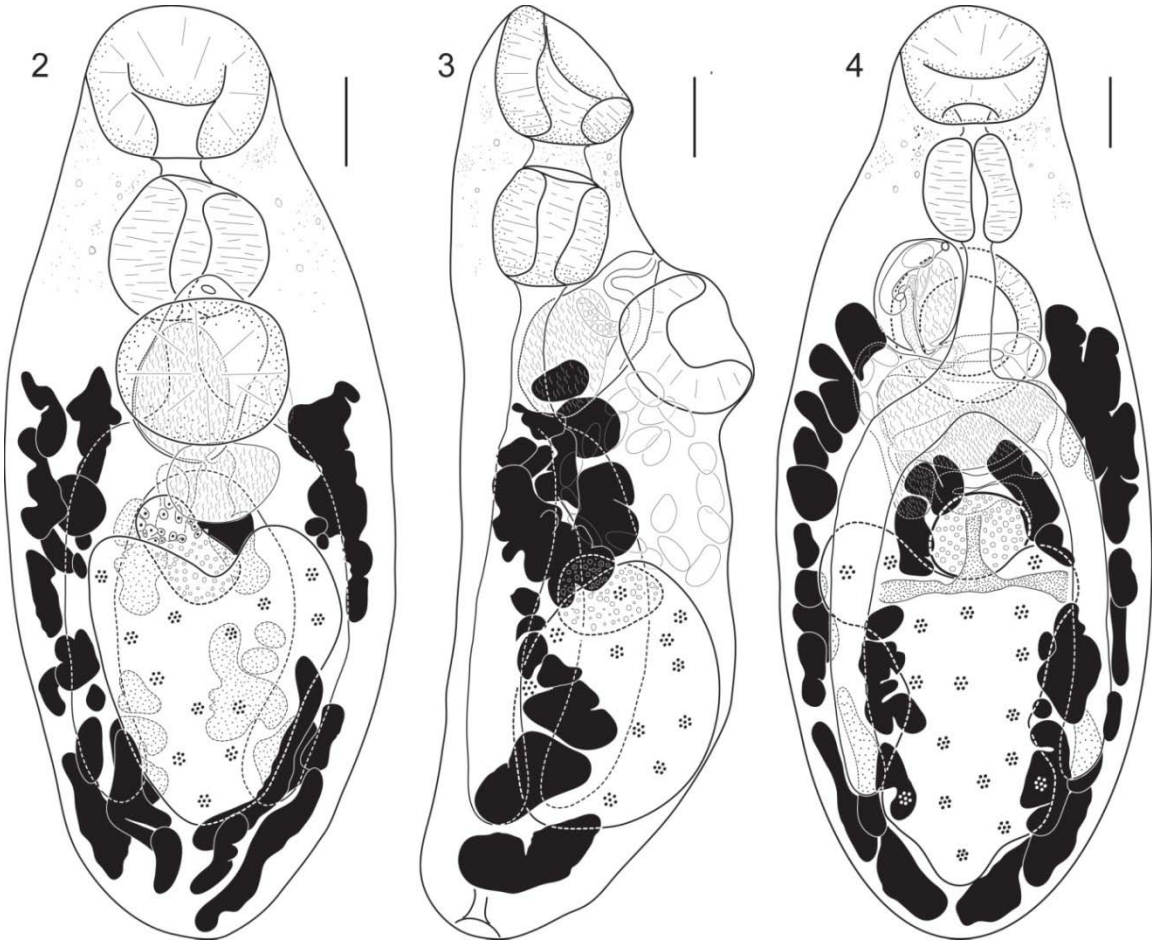


Figure V.2-4. Carassotrema ginezinskajae ex *Zacco platypus* 2. Ventral wholemount; 3. Lateral wholemount; 4. Dorsal wholemount, heat killed under coverslip pressure. *Scale-bars* 100µm.

Carassotrema ginezinskajae Kulakova & Ha Ky, 1976

Syns Asymphylodora ginezinskajae Ha Ky, 1969 (a dissertation referenced in Kulakova & Ha Ky, 1976) *nomen nudum, C. koreanum* of Moravec and Sey, 1989 in part.

Records

References: 1. Ha Ky (1969) (Cited in Kulakova & Ha Ky, 1976, apparently a thesis) 2. Kulakova and Ha Ky (1976) (used same specimens as Ha Ky, 1969) 3. Moravec and Sey (1989) 4. Present study.

Descriptions: 1; 2; 3; 4.

Distribution: China: Guangdong (4); Vietnam: Bac-kan (1, 2), Lao-cai (1, 2), Red River near Hanoi (3).

Definitive Hosts: *Distoechodon tumirostris* (4), no common name (Cyprinidae), *Hemiculterella sauvagei* Warpachowski (4), no common name (Cyprinidae); *Spinibarbus denticulatus* (Oshim) type host (1, 2), no common name (Cyprinidae); *Squaliobarbus curriculus* (Richardson) (3), barbel chub (Cyprinidae); and *Zacco platypus* (4), freshwater minnow (Cyprinidae).

Material Studied: *ex Zacco platypus* (Temminck & Schlegel, 1846) ex 011,012 Host 189; *ex Hemiculterella sauvagei* Warpachowski, 1888 ex033,034 immature DNA host 206; *ex Distoechodon tumirostris* Peters, 1881 ex184 immature DNA host 221. Purchased at fish market Shaoguan, Guangdong, China (24° 03' N, 113° 23' E); ex *Squaliobarbus curriculus* from Moravec and Sey, 1989 D-214-1a, D-214-1b; ex *Spinibarbus denticulatus* Micropictographs of type series RAS, No. 6-168 (5).

Supplementary Data: Table 1-3, Figures 2-4.

Remarks

Carassotrema ginezinskajae can be differentiated from congeners by pharynx, oral and ventral sucker being approximately equal in size and large relative to body size, distinct short prepharynx, pharynx occupying almost entire length between the ventral

and oral suckers, oesophagus terminating near mid-point of ventral sucker, testis occupying more than 1/3 of hindbody, caeca terminating near posterior margin of testis, genital pore near anterior margin of ventral sucker, and vitellarium composed of more numerous and smaller areas of follicles.

I do not accept the synonymy of *C. ginezinskajae* with either *C. koreanum* (Moravec & Sey, 1989) or *C. megapharyngus* (Overstreet & Curran, 2005). Moravec and Sey (1989) collected six specimens of *Carassotrema* from three species of fishes and concluded *C. koreanum* was highly variable with respect to the size of the suckers and pharynx, extent of vitellarium, and tegument spination. However Moravec and Sey (1989) did not state that any corresponded to *C. ginezinskajae*. The specimens Moravec and Sey (1989) collected were from preserved fish which may have confounded the identification of *Carassotrema* species. Overstreet and Curran (2005) did not accept the synonomy of *C. ginezinskajae* with *C. koreanum*, but considered it instead a synonym of *C. megapharyngus* on the basis of the literature. Examination of micropictographs of the type series of *C. ginezinskajae* leads me to consider my material as conspecific with those specimens and that *C. ginezinskajae* is not a synonym of *C. megapharyngus*.

Carassotrema heterorchis Wang and Pan, 1984

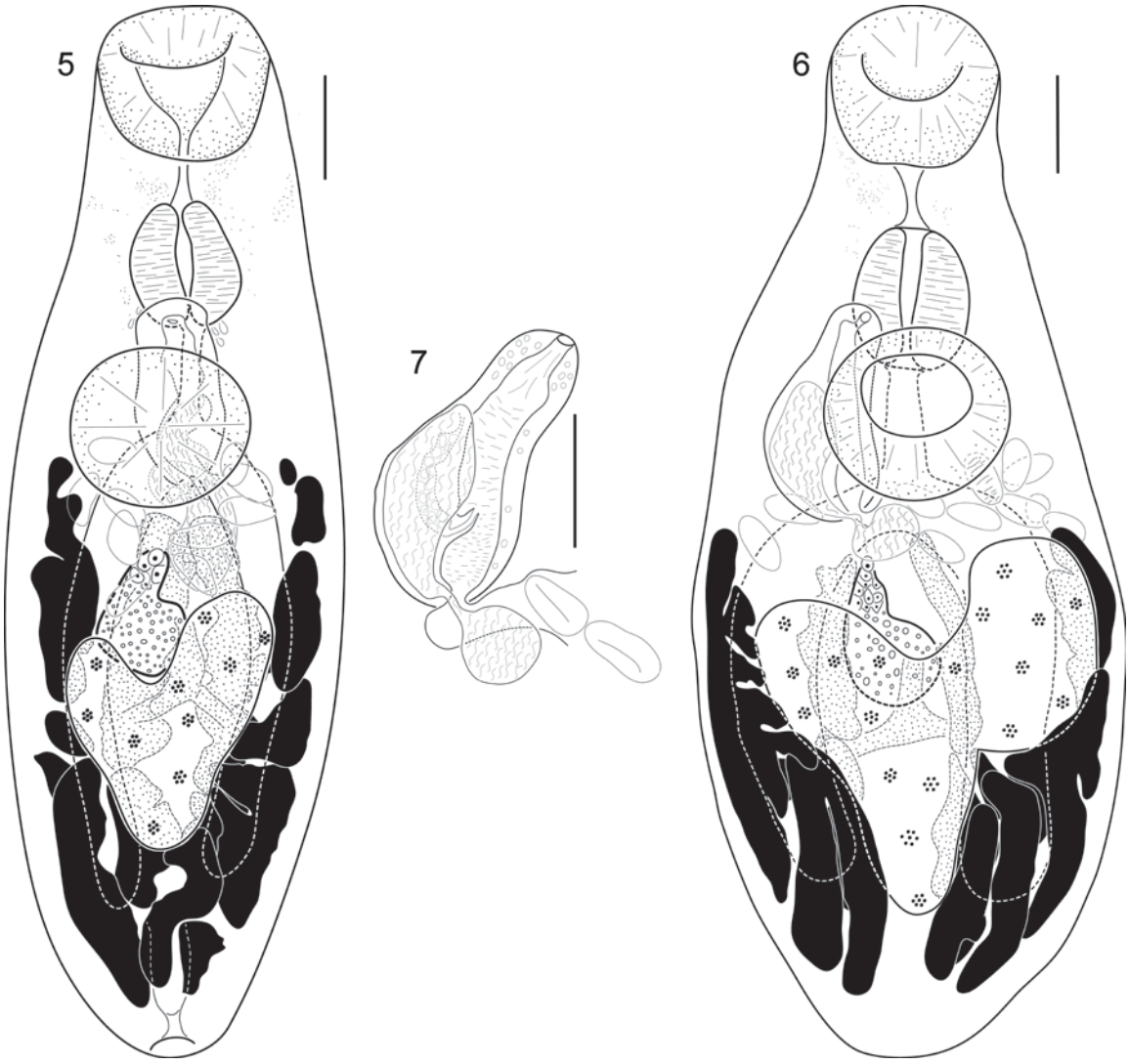
Syns Carassotrema koreanum of Long and Lee, 1958.

Records

References: 1. Long and Lee (1958) 2. Wang and Pan (1984), 3. Wang (1991) 4. Wang and Zhou (1993) 5. Present study

Descriptions: 1; 2; 3; 4; 5.

Distribution: China: Guangdon (5), Jiangsu (1), Jiangxi (4), Zhejiang (2, 3).



Figures V.5-7. Carassotrema heterorchis ex. *Spinibarbus hollandi* 5. Ventral wholemount; 6. Ventral wholemount, heat killed while under coverslip pressure; 7. Hemaphroditic sac of specimen heat killed while under coverslip pressure. *Scale-bars* 100µm.

Definitive Hosts: *Carassius auratus* (Linnaeus) (1) goldfish (Cyprinidae); *Chanodichthys erythropterus* (Basilewsky), predatory carp (Cyprinidae) / *Culter alburnus* Basilewsky (1), no common name (Cyprinidae); *Chanodichthys mongolicus* Basilewsky (1),Mongollian redfin (Cyprinidae); *Ctenopharyngodon idella* (Valenciennes) (type host) (1, 2, 3, 4), grass carp (Cyprinidae); *Cyprinus carpio* Linnaeus

(1), common carp (Cyprinidae); *Hemiculter leucisculus* (1) (Basilewsky), sharpbelly (Cyprinidae); *Hypophthalmichthys nobilis* Richardson (1), bighead carp (Cyprinidae); *Hypophthalmichthys molitrix* (Valenciennes) (1), silver carp (Cyprinidae); *Megalobrama terminalis* (Richardson) (1), Black Amur carp (Cyprinidae); *Mylopharyngodon piceus* Richardson (1), black carp (Cyprinidae); *Spinibarbus hollandi* (Nichols) (5), no common name (Cyprinidae); *Squaliobarbus curriculus* (Richardson (1), barbel chub (Cyprinidae); and *Xenocypris davidi* (3), no common name (Cyprinidae).

Material Studied: ex *Spinibarbus hollandi* host 201 ex-002, ex-196, purchased at fish market Shaoguan, Guangdong, China (24° 03' N, 113° 23' E).

Supplementary Data: Figures 5-7, Table 1-3.

Remarks

Carassotrema heterorchis is differentiated from congeners by a Y-shaped testis, distinct prepharynx, pharynx very close or overlapping ventral sucker, caeca extending beyond testis, and genital pore in posterior pharyngeal region.

My nonflattened specimens differ from those of Wang and Pan (1984) and Wang and Zhou (1993) in that the testis does not show the extreme bifurcation, the vitellarium commences at the posterior margin of the ventral sucker rather than posterior to ventral sucker, and the oesophagus extends to the midventral sucker rather than to the posterior margin of ventral sucker. I think this is due to my specimens not being compressed at the time of heat killing.

Long and Lee (1958) documented their finding of *Carassotrema* from 11 species of hosts, but their measurements of five specimens and their Figure 6 do not specify the hosts from which the data were derived. Although there appears to be a typographical

error associated with the measurement reported for the prepharynx and oesophagus, other measurements and Figure 6 by Long and Lee (1958) both correspond well to other published reports for *C. heterorchis*. The hermaphroditic duct of the illustrated specimen is extruded, and the description reports some spines on the hermaphroditic duct (Long & Lee, 1958). Although I have not examined these specimens, I postulate that the "spines" are actually glands associated with the hermaphroditic duct as seen in *Intromugil* (Pulis et al., 2013), *Malabarotrema*, and *Unisaccus* (Pulis, Chapter IV). In other species of haploporids

I have observed the extrusion of the hermaphroditic duct that appears have spine-like structures. Although the eggs measured by Long and Lee (1958) are unusually small, I still consider their specimens to be *C. heterorchis*. Across all the reports of *Carassotrema* species from China (Wang, 1973; Tang & Lin, 1979; Wang et al., 1983; Wang & Pan, 1984; Wang, 1991; Wang & Zhou, 1993), there is considerable variability among egg size of the specimens of the various authors; I suspect there are considerable differences in the individual *Carassotrema* species from different hosts.

Carasssotrema kui Tang and Lin, 1979

Syns Carasssotrema kui Tang and Lin, 1963; *Carassotrema koreanum* of Wang and Jiang, 1985.

Records

References: 1. Tang and Lin (1963), 2. Tang and Lin (1979), 3. Wang and Pan (1984) 4. Wang and Jiang (1985) 5. Wang (1991) 6. Wang and Zhou, (1993) 7. Present study.

Descriptions: 2; 3; 4; 5; 6; 7.

Distribution: China; Fujian (1, 2, 3), Guangdong (7) Hubei (3), Jaingxi (6), Sichuan (4), Zhejiang (5)

Definitive Hosts: *Acanthorhodeus chankaensis* (Dybowski) (6), Khanka spiny bitterling (Cyprinidae); *Bangana rendahli* (Kimura) (4), no common name (Cyprinidae); *Carassius auratus* (Linnaeus) (5), goldfish (Cyprinidae); *Ctenopharyngodon idella* (Valenciennes) (5), grass carp (Cyprinidae); *Distoechodon tumirostris* (7), *Hypophthalmichthys molitrix* (Richardson) (5), silver carp (Cyprinidae); *Hypophthalmichthys nobilis* (Valenciennes) (5), bighead carp (Cyprinidae); *Megalobrama terminalis*† (Richardson) (1, 2, 3, 6), Black Amur bream (Cyprinidae); *Rhodeus spinalis* Oshima (7), no common name (Cyprinidae); *Xenocypris davidi*† Bleeker (2, 3, 5, 6), no common name (Cyprinidae); *Xenocypris macrolepis* Bleeker, 1871 (3, 5, 6), yellowfin (Cyprinidae).

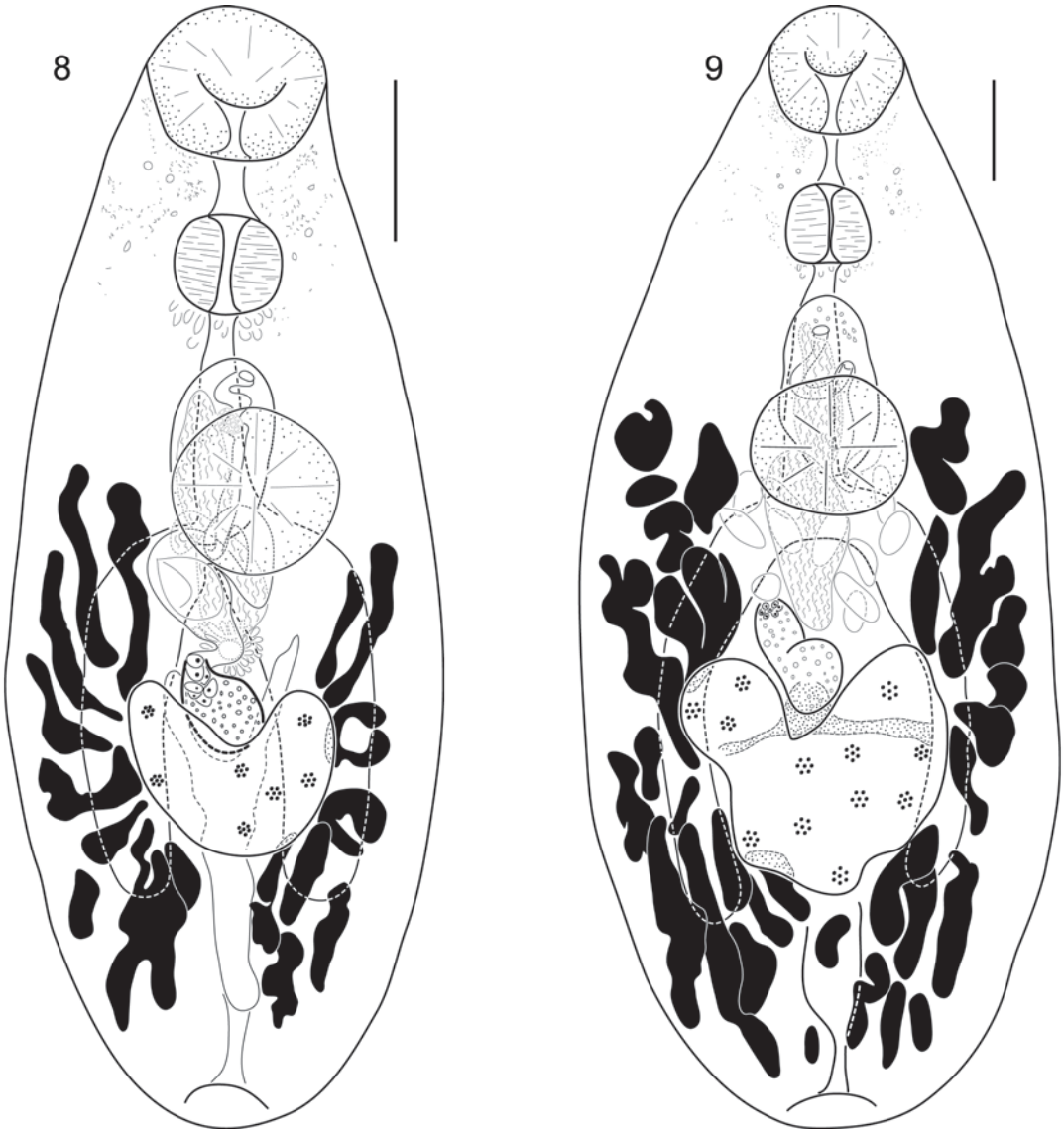
†Multiple species listed as definitive hosts in original description, but no host is designated as type host.

Material Studied: *ex. Rhodeus spinalis* USNPC: TBD GENBANK: TBD ex-007, ep-008, ep-193, ep-194, ep-195; *Distoechodon tumirostris* Peters, 1881 USNPC: TBD GENBANK: TBD ex-185 Purchased at fish market Shaoguan, Guangdong, China (24° 03' N, 113° 23' E).

Supplementary Data: Figures 8-9, Table 1-3.

Remarks

Carassotrema kui can be differentiated from congeners by a dorsoventrally flattened body, short prepharynx, long oesophagus terminating in posterior half of ventral sucker, wide U-shaped testis often irregular in outline, caeca terminating posterior to



Figures V.8-9. Carassotrema kui 8. Ventral wholemount ex. *Rhodeus spinalis*; 9. Ventral wholemount ex. *Distoechodon tumirostris*. *Scale-bars* 100µm.

testis, hermaphroditic sac terminating prior to posterior margin of ventral sucker, and vitellarium commencing in region of anterior half of ventral sucker.

I conclude that the specimens of *C. koreanum* of Wang and Jiang (1985) from *Bangana rendahli* in Sichuan, China, are *C. kui*. Both the description and figure

correspond with those in other reports (Tang & Lin, 1979; Wang & Pan, 1984; Wang, 1991; Wang & Zhou, 1993, present study) of *C. kui*, including the U-shaped testis, flattened body, dendtritic vitellarium (compared to other species of *Carassotrema*), hermaphroditic sac terminating prior to posterior margin of ventral sucker, and pharynx smaller than suckers in large specimens.

Carasssotrema lemellorchis Wang 1973

Synonyms: *Carassotrema lamellorchis* (*sic*.) Wang, 1973 Figure 27; *Carassotrema wui* Tang and Lin, 1963 *nomen nudum; Carassotrema wui* Tang and Lin, 1979.

Records

References: 1. Tang and Lin, (1963) 2. Wang (1973) 3. Tang and Lin (1979) 4. Wang et al. (1983) 5. Wang and Pan (1984) 6. Wang (1991) 7. Wang and Zhou, (1993).

Descriptions: 2; 3; 5; 6; 7.

Distribution: China: Fujian (1, 3, 5), Guangdong (5), Hubei (2, 5), Hunan (5), Jiangsu (4), Jiangxi (7), Zhejiang (6).

Definitive Hosts: *Acheilognathus taenianalis* (Günther) (7), no common name (Cyprinidae) *Carassius auratus* (Linnaeus) (3, experimental), goldfish (Cyprinidae); *Chanodichthys dabryi*† (Bleeker) (2), humpback (Cyprinidae); *Chanodichthys erythropterus* (Basilewsky), predatory carp (Cyprinidae)/ *Culter alburnus* Basilewsky†* (2, 4, 5, 6, 7), no common name (Cyprinidae); *Chanodichthys mongolicus* (Basilewsky)† (2), Mongolian redfin (Cyprinidae); *Ctenopharyngodon idella* (Valenciennes) (4, 5, 6, 7), grass carp (Cyprinidae); *Cyprinus carpio* Linnaeus (6), common carp (Cyprinidae); *Hemiculter leucisculus* (Basilewsky) (4, 6, 7), sharpbelly (Cyprinidae);

Hypophthalmichthys moltrix (Valenciennes) (6), silver carp (Cyprinidae); *Hypophthalmichthys nobilis* (Richardson) (6), bighead carp (Cyprinidae); *Parabramis pekinensis* (Basilewsky) (6), White Amur bream (Cyprinidae); *Plagiognathops microlepis* (Bleeker) (7), smallscale yellowfin (Cyprinidae); *Squaliobarbus curriculus* (Richardson) (1, 3, 4, 5, 6, 7,), barbel chub (Cyprinidae); and *Xenocypris davidi* (Bleeker) (6), no common name (Cyprinidae).

†Multiple species listed as definitive hosts in original description, but no host is designated as type host.

Intermediate host: *Stenothyra glabra* (Adams) (3), no common name (Gastropoda).

Remarks

Carassotrema lemellorchis can be differentiated from congeners by nearly equal suckers, short prepharynx, pharynx smaller than suckers, pharynx far anterior to ventral sucker, oesophagus terminating in anterior half of ventral sucker, tripartite testis Y-shaped, with three parts nearly equal in size, testis at about mid length of hindbody, hermaphroditic sac terminating near anterior margin of ventral sucker, genital pore noticeably anterior to ventral sucker, and vitellarium commencing in region of ventral sucker (based on description and illustration by Wang, 1973).

Carassotrema lemellorchis was first described by Wang (1973). Tang and Lin (1979) considered *C. lemellorchis* a synonym of *C. wui* Tang and Lin, 1963. *Carassotrema wui* Tang and Lin, 1963 was used in an abstract that listed only the name and name of the host, with Tang and Lin (1979) presenting the first description using that name in Tang and Lin, 1979. In 1973 Wang (1973) provided the first use of *C.*

lemellorchis with an accompanying description and illustration, but as *C. lemellorchis* Wang, 1964, apparently resulting from a delay in publication (Wang & Pan, 1984). *Carassotrema wui* Tang and Lin, 1963 is a *nomen nudum* and *C. lemellorchis* predates the 1979 description of *C. wui*. I consider *C. wui* Tang & Lin, 1963, *C. wui* Tang & Lin, 1979, and *C. lemellorchis* Wang, 1964 as synonyms of *C. lemellorchis* Wang, 1973. Additionally, in Wang (1973) *lemellorchis* is used for the description that occurs on page 184, and subsequently occurs at plate CVI Figure 27 as *C. lamellorchis*, a spelling that has been used by some subsequent authors. I consider *C. lemellorchis* Wang, 1973 as the accepted authority and spelling for this species.

Carassotrema lemellorchis and its synonyms have been reported numerous times in the literature, and these reports may represent more than one species (see remarks for *Carassotrema* sp. 1 and sp. 2).

Carassotrema megapharyngus Wang, 1973

Synonyms: *Carassotrema megapharyngus* Wang, 1964 *nomen nudum*; *C. heterosacca* Pan, 1965 (apparently a thesis) listed in Wang and Pan, 1984.

Records

References: 1. Wang (1973) 2. Tang and Lin (1979) 3. Wang et al. (1983) 4. Wang and Pan (1984) 5. Wang (1991) 6. Wang and Zhou, (1993).

Descriptions: 1; 2; 4; 5; 6.

Distribution: China: Guangdong (4), Hubei (1, 4), Fujian (2, 4), Jiangsu (3), Jiangxi (6), Zhejiang (5).

Definitive Hosts: *Acheilognathus taenianalis*† (Günther) (1), no common name (Cyprinidae); *Carassius auratus* (Linnaeus) (5), goldfish (Cyprinidae); *Crenimugil*

crenilabis (Forsskål, 1775), fringelip mullet (Mugilidae) (4), *Ctenopharyngodon idella* (Valenciennes) (4,5), grass carp (Cyprinidae); *Hemibarbus maculatus* Bleeker (3), spotted steed (Cyprinidae); *Hemiculter leucisculus* (Basilewsky) (5), sharpbelly (Cyprinidae); *Hypophthalmichthys moltrix* (Valenciennes) (5), silver carp (Cyprinidae); *Parabramis pekinensis* (Basilewsky)† (1, 3, 4, 5, 6), White Amur bream (Cyprinidae); *Megalobrama amblycephala* Yih (4,5), Wuchang bream (Cyprinidae); and *Megalobrama terminalis* (Richardson)† (1, 2, 4, 5,6), barbel chub (Cyprinidae).

†Multiple species listed as definitive hosts in original description, but no host is designated as type host.

Remarks

Carassotrema megapharyngus can be differentiated from congeners by indistinct prepharynx, pharynx larger than oral and ventral suckers, oesophagus terminating near posterior margin of ventral sucker, testis small, triangular, with shallow depression anteriorly, hermaphroditic sac terminating near mid-ventral sucker, genital pore noticeably anterior to ventral sucker, and vitellarium sparse, commencing in region of ventral sucker.

The original description of *C. megapharyngus* listed the authority as Wang, 1964, apparently originating from a delay in publication. The article was published in 1973 making the authority Wang, 1973 (Wang & Pan, 1984).

Carassotrema philippinense Machida, 1996

Records

References: 1. Machida (1996)

Distribution: Philippines: Mactan (1), Palawan (1).

Descriptions: 1.

Definitive Hosts: *Mugil cephalus* Linnaeus, flathead grey mullet (Mugilidae). Type host (1).

Material Studied: NSMT-P1-3939 1/2, 3939 2/2, 3525 2/3, 3525 3/3

Remarks

Carassotrema philippinense can be differentiated from congeners by the ventral sucker larger than pharynx, pharynx larger than oral sucker, distinct prepharynx, sac-like caeca and genital pore anterior to ventral sucker in the pharyngeal region.

The specimens of Machida (1996) were killed under coverslip pressure, which may make the caeca appear more saccular than they would be in life. Additionally, the testis shape may be ambiguous (in some specimens it is crushed and almost follicular), but in all likelihood is ovate in life; I tentatively retain this species within *Carassotrema*. Overstreet and Curran (2005) suggested this species may be conspecific with *Platydidymus flecterotestis*, but I do not support that assertion. *Platydidymus flecterotestis* possesses a crenulated oesophagus, H-shaped caecal arrangement, proportionally smaller ventral sucker, and U- to V-shaped testis, none of which appear in Machida's specimens. Pending molecular evidence and new non-flattened specimens, I retain *C. philippinensis* within *Carassotrema*.

On slide 3939 2/2 there is one specimen that does not appear to be conspecific with *C. philippinense*. The species possesses a very small ventral sucker and pharynx relative to body size. That specimen is marked on the slide with a illegible black mark, whereas the specimens that appear to have been used in the original description are

marked in blue. I, therefore, do not believe it was used for the original description by Machida (1996).

Carassotrema pterorchis Wang, 1973

Records

References: 1. Wang (1973) 2. Wang and Pan (1984) 3. Wang (1991) 4. Wang and Zhou (1993) 5. Present study.

Descriptions: 1; 2; 3; 4; 5.

Distribution: China: Guangdong (5), Hubei (1, 2), Jiangsu (2), Jiangxi (2, 4), Zhejiang (2, 3).

Definitive Hosts: *Carassius auratus* (Linnaeus) (4), goldfish (Cyprinidae); *Chanodichthys erythropterus* (Basilewsky), predatory carp (Cyprinidae) / *Culter alburnus* Basilewsky* (3), no common name (Cyprinidae); *Ctenopharyngodon idella* (Valenciennes)† (1, 2, 3, 4), grass carp (Cyprinidae); *Cyprinus carpio* Linnaeus (5), common carp (Cyprinidae); *Megalobrama terminalis* (Richardson) (3), Black Amur bream (Cyprinidae); *Mylopharyngodon piceus* Richardson (3), black carp (Cyprinidae); *Parabramis pekinensis* (Basilewsky)† (1, 2, 3, 4), White Amur bream (Cyprinidae); *Squaliobarbus curriculus* (Richardson)† (1, 3, 4), barbel chub (Cyprinidae); *Xenocypris davidi* Bleeker (3), no common name (Cyprinidae); and *Tachysurus fulvidraco* (Richardson) (3), yellow catfish (Bagridae).

†Multiple species listed as definitive hosts in original description, but no host is designated as type host.

Material Studied: ex. *Cyprinus carpio* Intestine. Purchased at fish market Shaoguan, Guangdong, China (24° 03' N, 113° 23' E) ex-187, ex-189.

Supplementary Data: Figure 10, Tables 1-3.

Remarks

Carassotrema pterorchis can be differentiated from congeners by suckers nearly equal in size, oesophagus terminating near level of mid-ventral sucker, Y-shaped testis, hermaphroditic sac terminating prior to posterior margin of ventral sucker, and genital pore noticeably anterior to ventral sucker, near pharyngeal region.

Unfortunately, I did not kill any of my specimens under coverslip pressure, and the identification of my specimens is inferred in a large part from how I believe the organs would shift under pressure and in comparison to other species.

The original description of *C. pterorchis* listed the authority as Wang, 1964, apparently originating from a delay in publication (Wang & Pan, 1984).

Carassotrema schistorchis Wang and Pan, 1984

Records

References: 1. Wang and Pan (1984) 2. Wang S. (1991) 3. Wang and Zhou, (1993).

Distribution: China: Guangdong (1), Hubei (1), Hunan (1), Jiangxi (3), Zhejiang (1, 2).

Descriptions: 1; 2; 3.

Definitive Hosts: *Carassius auratus* (Linnaeus) (2), goldfish (Cyprinidae); *Ctenopharyngodon idella* (Valenciennes)† (1, 2), grass carp (Cyprinidae); *Hemiculter leucisculus* (Basilewsky) (2), sharpbelly (Cyprinidae); *Hypophthalmichthys molitrix* (Valenciennes)† (1, 2), silver carp (Cyprinidae); *Hypophthalmichthys nobilis*

(Richardson)† (1, 2, 3), bighead carp (Cyprinidae); and *Parabramis pekinensis* (Basilewsky) (2), White Amur bream (Cyprinidae).

†Multiple species listed as definitive hosts in original description, but no host is designated as type host.

Remarks

Carassotrema schistorchis can be differentiated from congeners by distinct, short prepharynx, oesophagus extending to ventral sucker level, testis with numerous clefts laterally, and vitellarium commencing near level of mid-ventral sucker.

The most notable feature of *C. schistorchis*, the elongate testis with numerous lateral clefts, is noticeable even in immature specimens (Wang & Pan, 1984, Plate II Figure 1.) making reliable diagnosis possible even in immature specimens (Wang & Pan, 1984).

Carassotrema sp. 1 present study.

Definitive Host: *Carassius auratus* (Linnaeus), goldfish (Cyprinidae); present work

Material Studied: *ex Carassius auratus* ex-623, purchased at fish market Shaoguan, Guangdong, China (24° 03' N, 113° 23' E).

Supplementary Data: Figs 11, Tables 1-3. ep-623.

Remarks

Carassotrema sp. 1 specimens although immature, can be differentiated from other specimens by the elongate body shape, relatively large suckers, pharynx compared to body width, Y-shape testis, and genital pore at the anterior margin of the ventral sucker. The specimens procured do not contain eggs, although the internal and external

seminal vesicles and uterus were filled with sperm. Molecular data allowed us to differentiate this species from other specimens I obtained for the present study. Even though they are immature they appear to be morphologically closest to *C. lemellorchis* or *C. koreanum*. Additional specimens of this species are needed to determine the specific identity of these specimens and if they are representative of fully mature specimens.

Carassotrema sp. 2 present study.

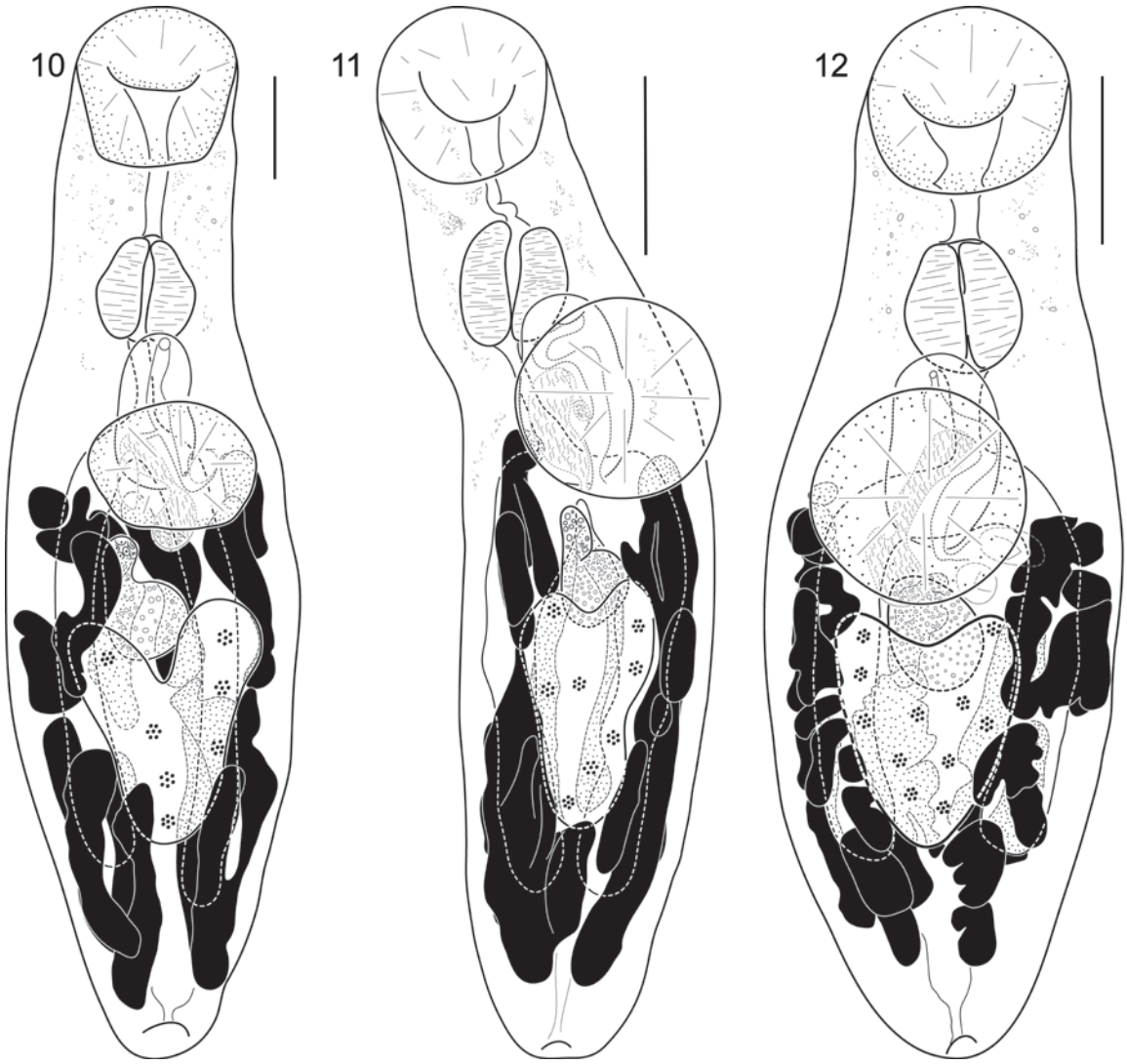
Definitive Host: *Carassius auratus* (Linnaeus), goldfish (Cyprinidae); present work.

Material Studied: *ex Carassius auratus* ex-026, ep-190, ep-191,ep-192, ep-188-*Cyprinus carpio* immature specimen. Purchased at fish market Shaoguan, Guangdong, China (24° 03' N, 113° 23' E).

Supplementary Data: Figures 12, Tables 1-3.

Remarks

The specimens obtained had a few, perhaps underdeveloped, eggs so I consider the specimens as immature. Therefore, I decline to identify them beyond genus. The hindbody of *Carassotrema* sp. 2 is proportionately short compared to body length, from my specimens reported here (other species) and Wang and Pan (1984) (*C. schistorchis* and *C. megapharyngus*); as species of *Carassotrema* mature they increase more in the hindbody proportion, making direct comparisons with fully mature specimens unreliable. Morphologically these specimens appear to be closest to *C. lemellorchis* or *C. koreanum*. Additional specimens of this species are needed to determine the specific identity of these specimens and if they are representative of fully mature specimens. Sequence data supports their inclusion within *Carassotrema*.



Figures V.10-12. Carassotrema pterorchis, Carassotrema sp. 1, and *Carassotrema* sp. 2
10. *Carassotrema pterorchis* ex *Cyprinus carpio* Ventral wholemount; 11. *Carassotrema* sp. 1, ex. *Carassius auratus* Ventral wholemount, external seminal vesicle not illustrated due to uterus being filled with sperm and indistinguishable; 12. *Carassotrema* sp. 2, ex. *Carassius auratus* Ventral wholemount. *Scale-bars* 100µm.

Carassotrema sp. 3 present study.

Definitive Host: *Chelon subvirdis* (Valenciennes) greenback mullet (Mugilidae); present work.

Material Studied: specimens from gall bladder of mullet very poor quality for morphological study. DNA. ex- *Chelon subvirdis* (Valenciennes) ep-618, ep-619.

Remarks

A number a degraded haploporids were procured from the gall bladder of a single *Chelon subvirdis* These specimens were dead prior to procurement and are not identifiable beyond the family level of Haploporidae Nicoll, 1914 in that they possess a single testis and hermaphroditic sac. Based on molecular data, I include these specimens as *Carassotrema* sp. 3. Because no species of *Carassotrema* of which I am aware are reported from the gall bladder of mullets, I encourage collection and description of this almost certainly unnamed species.

Platydidymus flecterotestis (Zhukov, 1971) Overstreet and Curran, 2005

Syns Haplotrema flecterotestis Zhukov, 1971, preoccupied; *Carassotrema flecterotestis* (Zhukov, 1971) Yu, Peng, and Liu, 2005

Records

References: 1. Zhukov (1971) 2. Li (1984) 3. Shen and Qiu (1995) 4. Overstreet and Curran (2005) 5. Yu, Peng, and Liu (2005).

Distribution: China: Liaoning (1); Bo Hai Sea (2,3); off west Tiawan (5).

Descriptions: 1; 3; 5.

Definitive Hosts: *Mugil cephalus* Linnaeus, (2) flathead grey mullet (Mugilidae); *Trachysurus brashnikowi* (Berg)† (1), Brazhnikov's catfish (Bagridae); *Liza*

haematocheila (Temminck & Schlegel)† (1, 3), so-iuy mullet (Mugilidae); and *Liza carinata* (Valenciennes) (4), keeled mullet (Mugilidae).

†Multiple species listed as definitive hosts in original description, but no host is designated as type host.

Material studied: RAS 11-199, 6 specimens on one slide ex. *Liza haematocheila*.

Remarks

Platydidymus flecterotestis possesses a crenulated oesophagus, H-shaped intestine, internal seminal vesicle occupying most of the hermaphroditic sac, and weakly organized vitellarium. These characters, in combination, prevent placement in *Carassotrema*. Caecal shape and nature is an important generic character and for this species has not been considered as a generic character (Chapter IV).

Amendments to *Platydidymus* Overstreet & Curran, 2005 diagnosis.

Caeca H-shaped. Oesophagus crenulated longer than prepharynx.

Carassotrema sp. n. 1 (Fiugures 13-18 Tables 2-4)

Type-host: *Nematalosa come* (Richardson) Western Pacific gizzard shad (Clupedae).

Site: Intestine.

Type-locality: Boat ramp south of Zilzie, Queensland, Australia, (23° 17' 55” S 150° 46' 00" E).

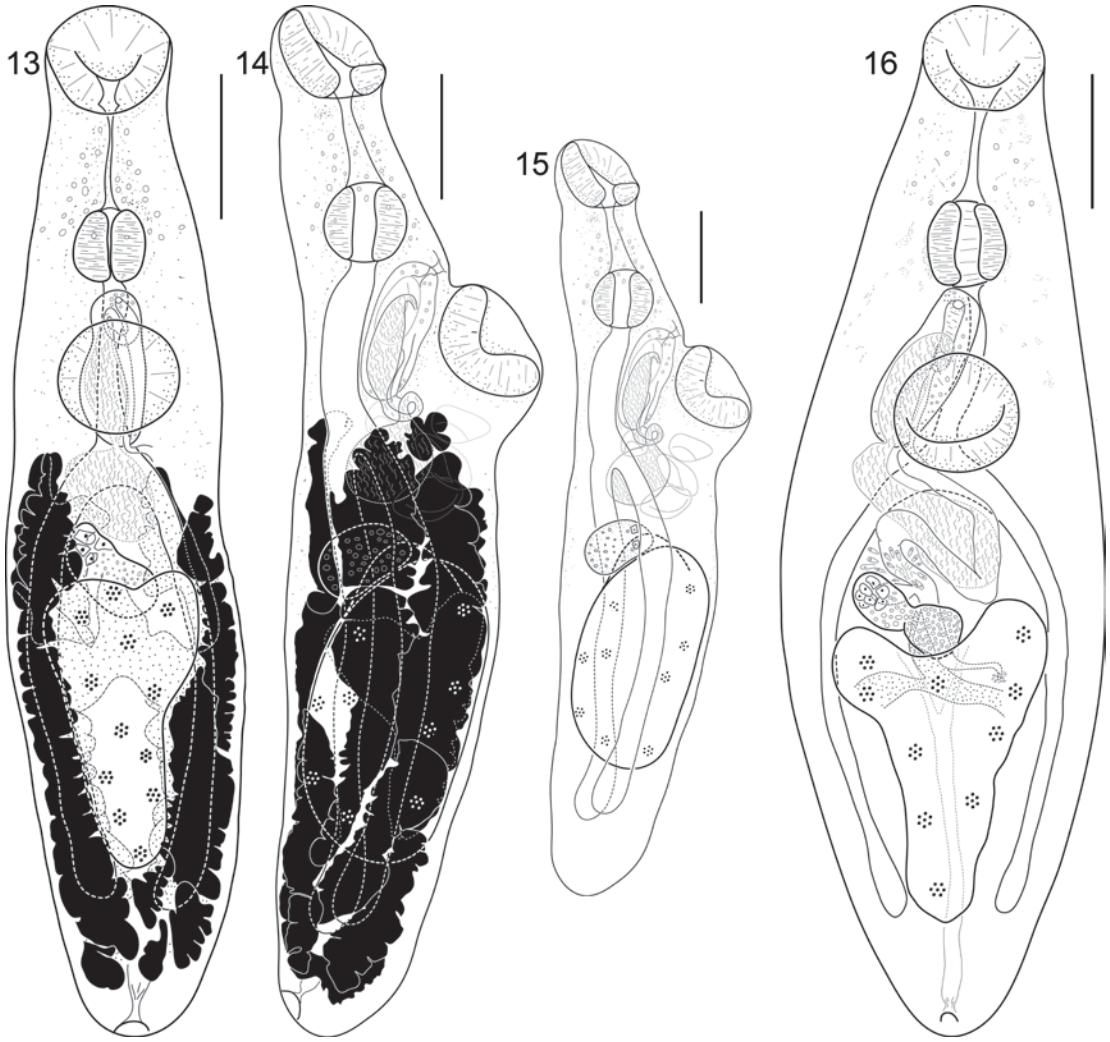
Other-locality: outlet from Causeway Lake, Queensland, Australia, (23° 12' 00” S 150° 47' 21" E).

Prevalence: Boat ramp south of Zilzie >50 from 12 *N. come* ~15 cm total length; Cause way lake outlet - 0 from 3 *N. come* ~22 cm total length; 5 from 2 *N. come* ~10 cm total length.

Material studied: QM TBD, USNPC TBD; Representative DNA sequence: partial 18S, entire ITS region, partial (D1-D3) 28S: representative sequence, GenBank accession no. TBD, from 4 identical sequences from Zilzie boat ramp, QLD.

Description: 12 specimens measured, range followed by mean in parentheses. Body fusiform, widest near midbody, 608-835 (741) long, 153-232 (184) wide, width 22-28% of body length (BL). Eye-spot pigment diffused, most prominent in forebody. Tegument spined, spines ~3-4 long, spines becoming smaller and more sparse toward posterior end of body. Oral sucker terminal, 56-80 (70) long, 63-98 (86) wide, width 38-53 (47%) of body width (BW). Ventral sucker slightly elevated, 69-91 (84) long, 71-106 (89) wide, width 45-51 (48%) of BW. Forebody 179-238 (213) or 27-30 (29%) of BL, hindbody 362-506 (448) or 59-62 (60%) of BL. Ventral sucker length 104-130 (120%) of oral sucker length, width 95-135 (104%) of oral sucker width. Prepharynx 63-76 (68) long. Pharynx muscular, globular, 48-64 (58) long, 49-63 (58) wide, length 70-102 (86%) of prepharynx length. Oesophagus 80-124 (102) long, bifurcation near posterior margin of ventral sucker, 255-331 (298) from anterior end of body, 37-43 (40%) of BL. Caeca long, tubular, ending blindly, 56-119 (84) from posterior end of body, postcaecal space 9-16 (12%) of BL.

Testis medial, Y-shaped, widest in anterior third, 197-280 (241) long, 96-139 (118) wide, width 43-54 (49%)of length, 69-104 (89) from ventral sucker, 83-174 (122) from posterior end of body, post-testicular space 13-21 (16%) of BL. Hermaphroditic sac,



Figures V.13-16. Carassotrema n. sp. 1 ex. *Nematalosa come*. 13. Holotype ventral wholemount; 14. Lateral wholemount; 15. Lateral wholemount without vitellarium; 16. Ventral wholemount of specimens killed while under coverslip pressure, without vitellarium which commences at posterior margin of ventral sucker. *Scale-bars* 100µm.

medial, dorsal to ventral sucker, at reflex angle to body plane, 88-116 (104) long, 37-50 (45) wide, containing internal seminal vesicle, 41-74 (56) long, 15-34 (25) wide, male duct arising from anterior internal seminal vesicle, prostatic bulb not prominent, only a few cells of a different type than that of male duct, joins with female duct to form hermaphroditic duct, lining of female and hermaphroditic duct smooth. Genital pore

medial, anterior to ventral sucker. External seminal vesicle sac-like, 58-98 (81) long, 43-68 (54) wide, duct from internal seminal vesicle often turns anteriorly overlapping posterior end of hermaphroditic sac, or with small loop, before turning posteriorly.

Ovary medial, often elongate, nestled in indent between lobes of testis, main portion with smallest follicles dorsal, with uterus emerging from anterior ventral portion, contiguous with testis, usually oriented near vertical, 76-98 (85) long by 41-64 (52) wide, 47-85 (61) posterior to ventral sucker. Ootype surrounded by Mehlis' gland situated ventral and anterior to ovary. True seminal vesicle absent. Laurer's canal present; pore dorsal in ovarian region. Vitellarium extensive, follicles plate-like sections, commencing near ventral sucker margin, although in minority of specimens vitellarium commences at posterior margin of ventral sucker, confluent posterior to testis. Uterus occupying space between testis and ventral sucker, direct without extraneous loops, proximal end filled with sperm, distal end without evidence of metraterm. Eggs few (1-14 from 11 ovigerious specimens, average 5, median 3), 54-62 × 29-32 (total of 10 eggs measured), terminal eggs not containing miracidium with pigmented eye-spots.

Lymphatic tubes in forebody. Excretory pore terminal. Excretory bladder Y-shaped, bifurcating in ovarian region with arms reaching to ventral sucker.

Remarks

Based on the combination of morphological features including diffused eye-spot pigment, spined tegument, distinct oesophagus, caeca tube-like terminating posterior to the testis, Y-shaped testis, possession of a hermaphroditic sac, sac-like external seminal vesicle, uterus confined to area between testis and hermaphroditic sac, extensive vitellarium commencing near the ventral sucker extending into the postesticular space,

and Y-shaped excretory vesicle, *C*. n. sp. 1 is consistent with the diagnosis of *Carassotrema*.

Carassotrema koreanum from freshwater cyprinid host differs from *C*. n. sp. 1 by is larger size, proportionately longer prepharynx, and vitellarium commencing near the mid ventral sucker. The figures of Tang and Lin (1979) show the vitellarium extending anteriorly beyond the ventral sucker and a proportionally smaller testis. Figure 4 (of a naturally dead specimen) by Park (1938) shows the vitellarium confined to the area dorsal to the testis, and the testis located noticeably ventral rather than middle body depth.

C. bengalense differs from *C*. n. sp. 1 by the shape of the body being bottle shaped, vitellarium commencing anterior to the midline of the ventral sucker, longer prepharynx, caecal bifurcation in region of ventral sucker, and elongate testis with ovary situated anterolateral to the testis (Rekharani & Madhavi, 1985; Shameem & Madhavi, 1991)

C. cluanodonae described from the clupeid *Konosirius punctatus* differs from *C*. n. sp. 1 by the shorter prepharynx, proportionately longer forebody, hermaphroditic sac not extending posterior to the ventral sucker, elongate entire testis, and ovary antero-sinistral (Liu, 2003).

C. estuarinum, hosted by the gray mullet *Mugil cephalus*, differs from *C*. n. sp. 1 by being a larger, wider worm, with a weakly Y-shaped to irregularly shaped testis, testis width 3/4 or greater of length, post-testicular space greater than 1/4 of BL, distinctly longer male duct, and vitellarium more densely developed with smoother margins (Tang & Lin, 1979).

Table V.1

Supplementary data for species of Carassotrema collected for this study. Measurements of wholemount non-flattened dorsal or ventral mounts.

Carassotrema sp. N measured- *Host*	*estuariunum* 2-*Mugil cephalus*	*ginezinskajae* 6-*Zacco platypus*	*heterorchis* 5-*Spinibarbus hollandi*	*kui* 4-*Rhodeus spinalis*, 4-*Distoechodon tumirostris*
Body L. (BL)	1459-1462	803-1202	906-1123	533-1130
Body W.	462-524	345-503	304-431	221-482
% of BL	32-36%	39-45%	32-41%	37-51%
Forebody	404-409	246-367	304-358	190-379
% of BL	28%	28-31%	30-37%	30-40%
Hindbody	901-906	418-665	452-607	230-616
% of BL	62%	52-56%	48-55%	43-55%
Body Spine L				
Oral Sucker (OS) L.	142-150	132-178	130-159	76-126
OS W.	166-168	161-209	147-173	83-132
Ventral sucker (VS) L.	147-156	143-190	128-164	88-148
VS W.	156-173	157-233	148-191	97-161
VSL / OSL %	98-110%	102-118%	89-116%	107-123%
VSW / OSW %	93-104%	98-115%	101-114%	98-144%
Prepharynx	69-70	4-26	49-73	0-44
Pharynx (PH) L	128-132	123-181	110-142	53-84
PH W	127-136	134-182	105-138	54-89
PH L / PH W	94-104%	88-112%	101-112%	92-108%
PH L / OS L	88-90%	91-104%	76-97%	62-73%
PH W / OS W	76-82%	73-96%	68-80%	61-70%
PH L / VS L	82-90%	79-102%	73-87%	56-63%
PH W / PH L	79-81%	68-89%	60-73%	48-63%
PH L / Prepharynx %	53-54%	3-17%	40-62%	0-54%
Oseophagus	215-218	92-163	93-126	92-213
Termination of Oesophagus	Posterior to VS	Near posterior margin of VS	Mid -VS	Posterior 1/2 of VS
Pre-caecal / BL	38%	30-46%	40-45%	42-49%
Post-caecal / BL	17%	10-19%	9-13%	11-20%
Caecal L / BL	49-50%	41-45%	41-47%	33-45%
Testis L	258-277	280-427	195-310	91-285
Testis W	211-221	255-350	138-307	110-363
Testis shape	irregular	Y	Y	U
Testis W / Testis L	76-86	80-92	79-106	93-166
Testis to VS distance	241-245	62-140	68-94	43-130
% BL	17%	8-13%	6-10%	5-12%
Post-testicular space	413-417	86-131	148-224	89-243
% BL	28-29%	9-13%	16-20%	17-24%
Ovary L	102-111	99-157	81-125	35-112
Ovary W	61-126	54-117	76-86	32-75
Ovary to VS distance	186-195	27-99	42-80	27-90
Testis to Ovary dist.	0	0	0	0
Hermaphroditic sac L	274-308	181-233	136-196	93-183
Hermaphroditic sac W	149-150	97-162	96-150	55-110
ISV L	205-233	107-157	75-141	51-118
ISV W	82-90	55-116	40-83	21-63

Table V.1 (continued).

Carassotrema sp.	*estuariunum*	*ginezinskajae*	*heterorchis*	*kui*
ESV L	126-189	119-221	61-143	54-179
ESV W	111-112	67-166	51-114	40-98
Vitellarium to VS	42-54 posterior	Mid VS	Posterior 1/3 to 34 posterior	Anterior 1/2
Egg L	57-63	58-67	57-65	51-63
Egg W	29-34	27-34	28-33	26-41

C. ginezinskajae differs from *C.* n. sp. 1 by the larger pharynx, shorter prepharynx, and vitellarium commencing further anteriorly (Kulakova & Hy Ka, 1976).

C. heterorchis differs from *C.* n. sp. 1 by the wider body, the shape of testis being Y-shaped approaching V-shaped with deep recess in the anterior forks, the anterior end of the testis reaching the region of the ventral sucker, and genital pore in the pharyngeal level (Wang, 1973).

C. kui differs from C. n. sp. 1 by having a pyriform body shape, a vitellarium that commences in the region of the anterior margin of the oral sucker, hermaphroditic sac terminating anterior to the posterior margin of the ventral sucker, and a wider U-shaped testis (Tang & Lin, 1979; Wang & Pan, 1984; Wang, 1991).

C. lemellorchis differs from *C.* n. sp. 1 by several features, including the hermaphroditic sac terminating prior to the ventral margin of the ventral sucker, shorter prepharynx, testis being more strongly Y-shaped with deeper anterior cleft, and greater width to length ratio (Wang, 1973; Tang & Lin, 1979; Wang & Pan, 1984; Wang, 1991).

C. megapharynx differs from *C.* n. sp. 1 by the much larger pharynx, shorter prepharynx, and proportionately smaller testis (Kulakova & Hy Ka, 1976; Tang & Lin, 1979; Wang & Pan, 1984; Wang, 1991).

Table V.2

Supplementary data for species of Carassotrema collected for this study. Measurements of wholemount non-flattened dorsal or ventral mounts.

Carassotrema sp. N measured- *Host*	*pterorchis* 4-*Cyprinus carpio*	Sp. 1 2-*Carassius auratus*	Sp. 2 6-*Carassius auratus*	n. sp. 1 n. sp. 12-*Nematalosa come*
Body L. (BL)	1002-1031	619-680	535-651	608-835
Body W.	272-313	145-165	182-217	153-232
% of BL	27-31%	23-24%	31-35%	22-28%
Forebody	355-400	182-201	204-249	179-238
% of BL	35-40%	29-30%	33-41%	27-30%
Hindbody	457-517	325-372	233-326	362-506
% of BL	46-51%	53-55%	41-50%	59-62%
Body Spine L				
Oral Sucker (OS) L.	129-153	92	107-121	56-80
OS W.	144-155	95-100	109-118	63-98
Ventral sucker (VS) L.	124-160	105-114	104-117	69-91
VS W.	136-169	115-135	114-128	71-106
VSL / OSL %	88-113%	114-124%	93-106%	104-130%
VSW / OSW %	94-111%	121-135%	101-113%	95-135%
Prepharynx	76-90	29-32	20-33	63-76
Pharynx (PH) L	97-109	67-76	75-84	48-64
PH W	88-97	62-67	66-74	49-63
PH L / PH W	100-124%	108-113%	104-120%	95-107%
PH L / OS L	70-75%	73-83%	69-74%	71-91%
PH W / OS W	59-64%	65-67%	59-66%	58-89%
PH L / VS L	65-80%	59-72%	68-76%	56-76%
PH W / PH L	57-65%	50-54%	54-62%	58-69%
PH L / Prepharynx %	70-93%	42-43%	26-42%	70-102%
Oseophagus	61-82	60-69	34-52	80-124
Termination of Oesophagus	Anterior 1/2 of VS	Posterior 1/2 of VS	Mid-VS	Near posterior margin of VS
Pre-caecal / BL	39-43%	41%	40-43%	37-43%
Post-caecal / BL	11-19%	13-17%	14-20%	9-16%
Caecal L / BL	41-47%	52-53%	39-55%	43-53%
Testis L	200-243	133-144	124-155	197-280
Testis W	141-190	69-74	99-127	96-139
Testis shape	Y	Y	Y	Y
Testis W / Testis L	71-79	48-56	79-89	43-54
Testis to VS distance	48-66	58-75	0-20	69-104
% BL	5-7%	9-11%	0-3%	9-14%
Post-testicular space	200-222	131-160	103-177	83-174
% BL	19-22%	21-24%	17-29%	13-21%
Ovary L	91-122	69-90	58-71	76-98
Ovary W	59-98	39-44	38-69	41-64
Ovary to VS distance	0-48	5-15	0	47-85
Testis to Ovary dist.	0	0	0	0
Hermaphroditic sac L	145-170	110-113	89-110	88-116
Hermaphroditic sac W	68-92	65	51-78	37-50
Carassotrema sp.	*pterorchis*	Sp. 1	Sp. 2	n. sp. 1 n. sp.
ISV L	61-75	61-73	39-63	41-74
ISV W	29-33	22-27	29-35	15-34

Table V.2 (continued).

Carassotrema sp.	*pterorchis*	Sp. 1	Sp. 2	n. sp. 1 n. sp.
ESV L	69*	58*	59*	58-98
ESV W	50*	56*	35*	43-68
Vitellarium to VS	Posterior 1/2	Mid-VS	Mid-VS	Posterior 1/3 to 14 posterior
Egg L	60-70	NA	54-59†	54-60
Egg W	26-31		26-31†	29-32

C. philippinensis differs from *C.* n. sp. 1 in the following characters pharynx pyriform, ventral sucker width at least 160% of oral sucker width, testis round to elongate, and prostatic bulb large and distinct large in comparison to *C.* n. sp. 1 (Machida, 1996).

C. pterorchis, differs from *C.* n. sp. 1 by having a distinctly Y-shaped testis, with the three parts being nearly equal in size and oesophagus terminating near mid-ventral sucker level (Tang & Lin, 1979; Wang, 1991).

The larger *C. schistorchis* differs from *C.* n. sp. 1 by having an elongate testis with many lateral indents (Wang & Pan, 1984).

Platydidymus flecterotestis differs from *C.* n. sp. 1 by possession of an H-shaped intestine rather than the long tubular caeca *C.* n. sp. 1, crenulated rather than smooth oesophagus, possession of a comparatively large distinct prostatic bulb (type slide examined not clear in all specimens), and deeply cleft V-shaped testis (Zhukov, 1971; Overstreet & Curran, 2005; Yu et al., 2005).

Molecular Results.

I compared DNA sequence data from 9 species of *Carassotrema*. The fragment sequenced encompassed the 3' end of the 18S gene, the ITS region (ITS1 + 5.8S + ITS2) and *c*. 1,370 bp of the 5' end of the 28S gene. According to the available information on the sequenced contiguous fragment, these regions are generally suitable for discrimination of congeneric digenean species (e.g., Nolan & Cribb, 2005; Olson & Tkach, 2005; Tkach & Mills, 2011; Pulis et al., 2013; Pulis & Overstreet, 2013). Intraspecific variation was detected at one position in *Carassotrema* sp. 3 from the gallbladder of *C. subvirdis*. Lengths of the sequenced region used for species discrimination (when trimmed to the utilized portions) ranged from 2,359 for *C. kui* to 2,628 for *C.* n. sp. 1. The main discrepancies in length are largely attributed to indels in the ITS1 region. As such, the 5' end was not usefully alignable for the entire set of species. The ITS1 sequence lengths were 810 for *C.* n. sp. 1, 710 for *C.* sp. 3, 706 for *C. estuarinum*, 632 for *C. heterorchis*, 603 for *C. pterorchis*, 576 for *C. ginezinskajae*, 558 for *C.* spp. 1 and 2, and 544 for *C. kui*. Due to the variability in lengths only about 380 bp (aligned length) of the ITS1 were used for analysis. The intrageneric pairwise percent variation ranges are 0.5-10.1% in ITS1(*c*. 380 of 3' end), 0-1.9% for 5.8S, 0.4-7.4% for ITS2, and 0.7-6.0% for *c.* 1380 bp of 5' of 28S. The high end of these ranges found for *Carassotrema* are slightly higher than previous authors (Blasco-Costa, Balbueno et al., 2009; Pulis et al., 2013; Pulis & Overstreet, 2013) have found for other comparisons of congeneric haploporids. This is however the first time such a comparison was conducted with more than four species of a particular genus. *Carassotrema* n. sp. 1 consistently has

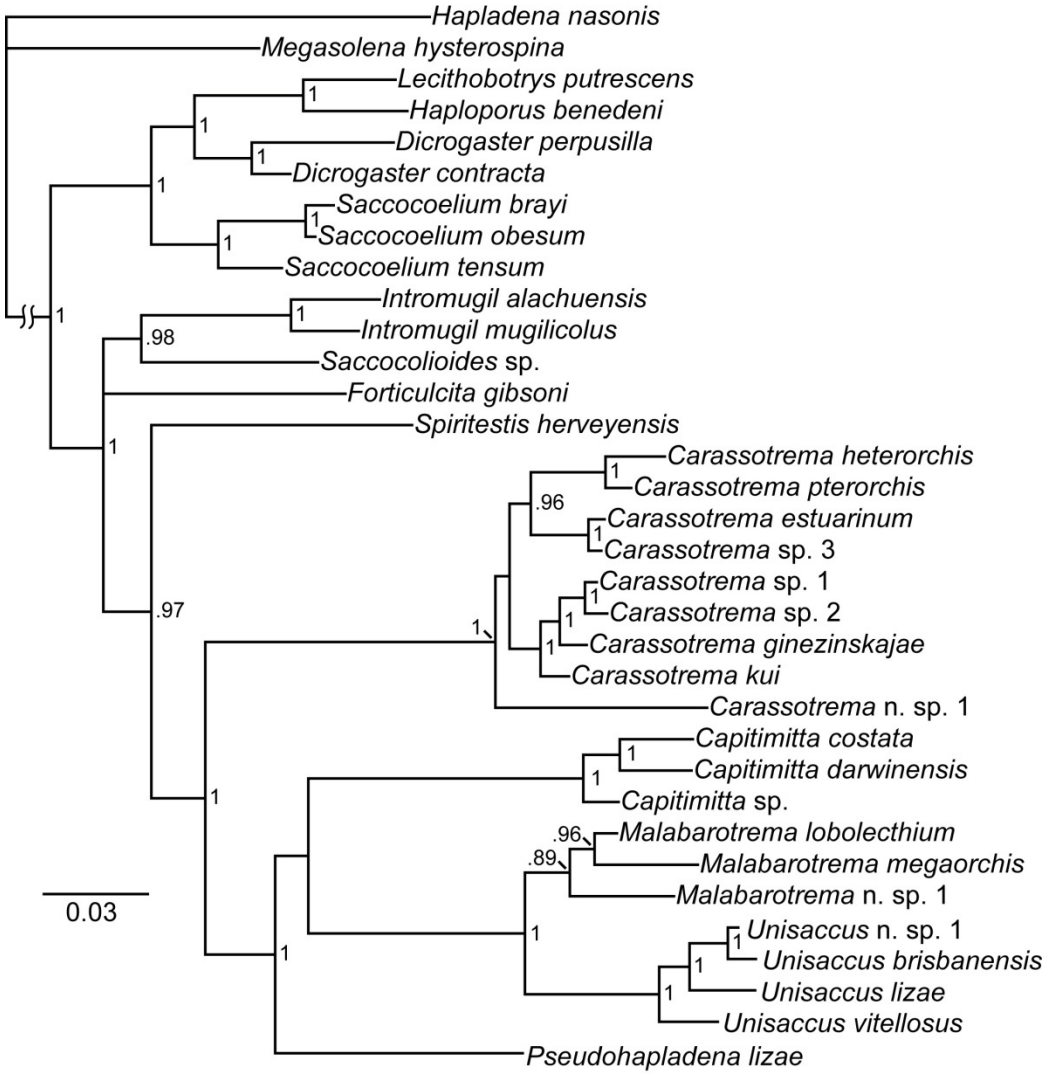


Figure V.17. Estimated position of *Carassotrema* within the Haploporidae 17. Phylogenetic tree resulting from Bayesian analysis (1,000,000 generations) of partial sequences of 28S rDNA gene. Posterior probabilities greater than 80 are shown.

above average values of variation when compared to the other species of *Carassotrema* treated here.

The BI analysis of the partial 28S rDNA gene sequences (Figure 17) included 15 genera of haploporids. The branch leading to nine species of *Carassotrema* formed a well supported monophyletic clade between *Spiritestis herveyensis* Pulis and Overstreet, 2013 and a clade formed by *Capitimitta* + *Paraunisaccoides* + *Unisaccoides* + *Unisaccus*. The

monophyletic *Carassotrema* species form three subgroups formed by *C.* n. sp. 1, *C. kui* + *C. ginezinskajae* + *C.* sp. 1 + *C.* sp. 2, and *C. heterorchis* + *C. pterorchis* + *C. estuarinum* + *C.* sp. 3. The BI tree of partial ITS region (partial ITS1, 5.8S, and ITS2) showed lower support overall and differed in topology (Figure 18); *C.* n. sp. 1 grouped internally with *C. estuarinum* + *C.* Sp.3 with C. heterorchis being basal to the remaining species of *Carassotrema*.

Discussion

The composition of Carassotrema and its geographic distribution has been controversial. For differing opinions see (Nasir & Gomez, 1976; Kohn et al., 1999; Overstreet & Curran, 2005; Yu et al., 2005). In this study I have provided sequence data for nine species that form a monophyletic clade that I recognize as species of *Carassotrema*. *Intromugil mugilicolus* originally described within *Carassotrema* is clearly not a close relative of *Carassotrema* species (Figure 17). Additionally, those species Overstreet and Curran (2005) removed from *Carassotrema* (*C. heterorchis, C.kui, and C. ginezinskajae* [as a synonym of *C. megapharyngus*]) and placed into *Platydidymus* are not supported as a natural group (Figures 17-18, Tables 2-3).

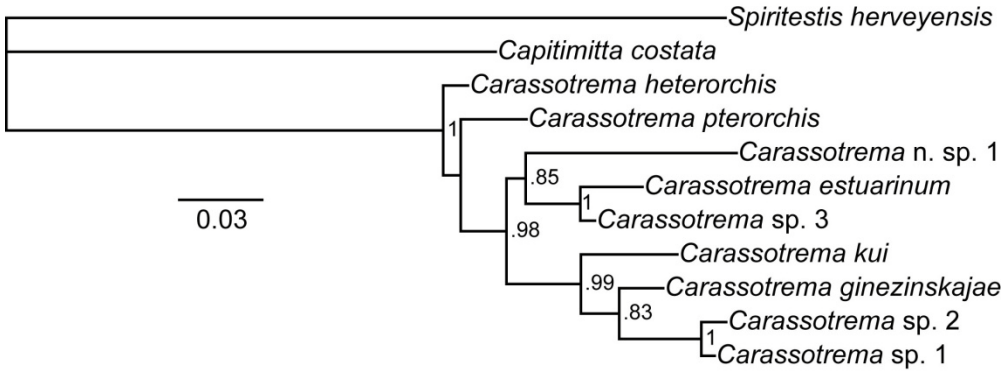


Figure V.18. Estimated relations of *Carassotrema* species 18. Phylogenetic tree resulting from Bayesian analysis (1,000,000 generations) of partial sequences of ITS region. Posterior probabilities greater than 80 are shown.

Table V.3

Percent variable sites based on pairwise sequence comparison of the 5.8S gene (above diagonal) and about 380 bases at the 3' end of the ITS1 region (below diagonal) of nuclear ribosomal DNA among 9 species of Carassotrema.

	Carassotrema estuarinum	*Carassotrema ginezinskajae*	*Carassotrema heterorchis*	*Carassotrema kui*	*Carassotrema pterorchis*	*Carassotrema* sp. 1	*Carassotrema* sp. 2	*Carassotrema* sp. 3	*Carassotrema* n. sp. 1
C. estuarinum	-	1.9	1.3	1.3	1.3	1.9	1.9	0.6	1.3
C. ginezinskajae	6.5	-	0.6	0.6	0.6	0	0	1.3	1.3
C. heterorchis	6.5	5.7	-	0	0	0.6	0.6	0.6	1.3
C. kui	7.3	2.3	6.0	-	0	0.6	0.6	0.6	1.3
C. pterorchis	8.6	6.2	3.9	6.8	-	0.6	0.6	0.6	1.3
C. sp. 1	6.0	1.6	5.5	3.1	6.0	-	0	1.3	1.3
C. sp. 2	6.0	1.6	5.5	3.1	6.0	0.5	-	1.3	1.3
C. sp. 3	2.6	5.7	6.0	6.0	7.3	5.2	5.2	-	1.9
C. n. sp. 1	9.7	8.4	8.6	7.6	10.6	8.6	8.6	8.4	-

Table V.4

Number of variable sites based on pairwise sequence comparison of the ITS2 region (below diagonal) and partial (c. 1300) 28S gene (above diagonal).

	Carassotrema estuarinum	*Carassotrema ginezinskajae*	*Carassotrema heterorchis*	*Carassotrema kui*	*Carassotrema pterochis*	*Carassotrema* sp. 1	*Carassotrema* sp. 2	*Carassotrema* sp. 3	*Carassotrema* n. sp. 1
C. estuarinum	-	3.1	4.1	2.8	3.3	2.9	3.1	0.6	5.1
C. ginezinskajae	7.0	-	4.3	4.1	3.3	1.1	1.2	3.1	4.7
C. heterorchis	4.2	4.2	-	1.5	1.9	1.7	1.2	4.3	6.0
C. kui	7.4	4.6	4.6	-	3.0	0.7	2.9	2.8	4.4
C.pterorchis	5.7	4.6	1.8	4.9	-	3.3	3.5	3.3	5.1
C. sp. 1	6.7	2.1	3.2	4.2	3.5	-	3.1	2.9	4.9
C. sp. 2	6.4	4.6	3.5	4.6	3.9	0.4	-	3.1	4.9
C. sp. 3	3.2-3.5	5.3	3.2	5.3	3.9	4.9	4.6-4.9	-	4.9
C. n. sp. 1.	6.6	5.2	3.8	5.2	4.2	4.2	4.2	4.2	-

* Of the two sequences generated for C. sp. 3 of the ITS2 there was an ambiguous base at the 78th position in one sequence and a T in the other sequence which is at an indel compared to most species; pairwise difference calculated for both alternatives.

Both levels of analysis identified the species I consider here as *Carassotrema* as a monophyletic group, albeit with differences in topology of the group (Figures 17-18). In both trees a clade composed of *C. kui*, *C. ginezinskajae*, *C.* sp. 1, and *C.* sp. 2 was recovered. The most notable difference between the two trees is the position of the new species *C.* n. sp. 1. In the 28S tree (Figure 17), *C.* n. sp. 1 is represented as sister to the remaining species of *Carassotrema* in the analysis. The less robust ITS tree (Figure 18) shows an internal clade with *C.* n. sp. 1 as sister to the a clade of *C. estuarinum* and *C.* sp. 3. Based on morphological features and definitive hosts infected I would have expected for *Carassotrema* species treated here to be divided into two divisions represented by a marine clade and a freshwater clade. Neither analysis supports that assumption. I must also note that material of *C.* sp. 3 was poor, so I do not have good specimens for morphological characterization. Also, based solely on morphological characterization of the species I collected, I would have expected *C. heterorchis*, *C. pterorchis*, *C.* sp. 1, and *C.* sp. 2 to group together rather than *C. kui* and *C. ginezinskajae* with *C.* sp.1 and *C.* sp. 2. In both trees (Figures 17-18), the second node in the generic clade has low support, probably indicative of rapid radiation at an early point in the history of the genus. More species and genes are needed to resolve these problems. While *Carassotrema* is a biologically cohesive group, there is still a disconnection between morphology and sequence data. The *C.* n. sp. 1 morphologically is closest to *C. estuarinum*, although they are certainly not each other's closest relative based on pairwise sequence divergence (Tables 2-3) and the trees built (Figure 17) of the species of Carassotrema with sequence data available. The pairwise difference between *C.* n. sp. 1 from the 28S sequence data shows higher divergence from other members of the genus, 4.4-6%.

Results of the BI analysis for the larger group of haploporids (Figure 17) confidently places the genus within what is currently considered the Waretrematinae (Pulis & Overstreet, 2013; Chapter IV). As molecular data for *Waretrema* is unavailable and *Unisaccus* has been transferred into the Waretrematinae, there is currently no morphologically based diagnosis for the subfamily and its limits are uncertain. With the addition of *Carassotrema* species to the public domain, I conclude that the generic synonymies of Nasir and Gomez (1976) are untenable as *Intromugil mugilicolus* and *Pseudohapladena lizae* are recovered as distinct from *Carassotrema* species. Several species that Overstreet and Curran (2005) had considered to be in *Skrjabinolecithum* have been transferred to *Malabarotrema* Zhukov, 1972 and *Unisaccoides* Martin, 1973 (Chapter IV). I consider that *Skrjabinolecithum spasskii* Belous, 1954 is likely a closer relative of *Carassotrema* than to *Malabarotrema* or *Pseudohapladena*.

Carassotrema n. sp. 1 appears to be furthest from the core of the genus as would be expected for a species the furthest removed geographically. Other geographically distant species are not available for molecular analysis, namely *C. philippinensis* and *C. bengalense*. The addition of these species to this data set would improve inferences as to the radiation of the genus. While I have made some inferences on the relations among the species of *Carassotrema* based on 28S rDNA data my purpose for this gene was primarily to place the genus within the Haploporidae. I also generated a BI tree based on the partial ITS region of the *Carassotrema* species (Figure 17), which differed in topology from the 28S tree. ITS showed a polytomy of *C. heterorchis*, *C. koreanum*, and a clade formed by the other seven species. *Carassotrema* n. sp. 1, rather than forming a clade by itself, showed a stronger affiliation to the *C. estuarinum* / *C*. sp. 3. The lengths

of the ITS 1 region of these three species are all significantly longer than the other six species, although trimmed of indels, which appeared to significantly affect the resulting alignment and, therefore, the BI generated tree. Using either the generated 28S or ITS trees, I revealed that Overstreet and Curran's (2005) transfer of several *Carassotrema* species *C. ginezinskajae* (which they considered a synonym of *C. megapharyngus*), *C. kui*, *C. megapharyngus*, and *C. heterorchis*)to the genus *Platydidymus* is unwarranted. My morphological study also shows that the testis shape of some species (most notably *C. heterorchis*) is highly dependent on the amount of pressure applied to the specimen when heat killed.

I do not know where the mullet from which *C. estuarinum* and *C.* sp 3 collected acquired the infection; while I expect that the ancestral *Carassotrema* was of a marine origin, *C. estuarinum* / *C.* sp. 3 may be a case of a secondary acquisition of a brackish host or alternatively multiple acquisitions of freshwater host from a basal marine *Carassotrema* species. The conflicting topology of the 28S and ITS trees (Figures 17, 18) shows that more study is required to determine relations of species of *Carassotrema*.

Taxonomy and systematics of *Carassotrema* would benefit from recollection of all Southeast Asian species from other host and major river drainages to determine how widespread these species are and if the individual species do show the wide host use and geographic range as indicated by myself and others. All my freshwater cyprinid specimens were from the same river drainage and constitute mostly new host records. I have provided molecular data, but I include the caveat that the worms collected may represent morphotypes rather than the species as originally described. Alternatively, China has a long history of aquaculture, and it is possible that species of *Carassotrema*

have been widely transported outside their native ranges. Collection of specimens for molecular analysis of population level genes may help to understand if these species have been transported by human activity or are widespread species with distinct lineages.

If all the records of *Carassotrema* synonyms, locations, and hosts are correct, the distribution and breadth of the freshwater cyprinid hosted species of *Carassotrema* are some of the most broadly distributed trematodes known. I suspect that there are numerous unnamed species, but the lack of type specimens severely limits making robust decisions about the patterns of diversification or host use in these species. While any one of these species could be imagined to have such a wide host use, the majority of them possessing such breadth of host use most likely indicates that the diversification of this genus in freshwater Asia is being driven primarily by first intermediate host use rather than definitive host use. Most other species of haploporids have been reported only at the time of their original description; thus, only one host is reported. In China there have been many reports of the genus from many localities and, thus, perhaps the host use is more a product of intensive sampling and other species in the family would benefit from the same broad examination of host. The most commonly reported hosts also are those fish which attain larger size and are of economic importance such as *Carassius auratus*, *Cyprinis carpio*, *Hypophthalmichthys molitrix*, *Hypophthalmichthys nobilis*, *Ctenopharyngodon idella*, and *Mylopharyngodon piceus*, thus giving them higher priority for examination for parasites than other, less economically important fishes.

CHAPTER VI

NEW GENUS 1 NEW SPECIES (TREMATODA: HAPLOPORIDAE) FROM THE SQUARETAIL MULLET *ELLOCHELON VAIGIENSIS* (ACTINOPTERYGII: MUGILIFORMES) FROM AUSTRALIA

Abstract

New genus 1 is proposed as monotypic for New genus 1 new species, a new species of trematode in the family Haploporidae. The new species is described from the squaretail mullet (*Ellochelon vaigiensis*) in Australia. The new genus differs from other known members of the family by having a cleft oral sucker, row of spines on the posterior opening to the mouth, wider than long caecum, thick-walled hermaphroditic sac, and cells lining the external seminal vesicle having large nucleated cells. The new genus is most similar to members of the Haploporinae in possessing in-utero eggs containing miracidium with eye-spots and reduced vitellarium. Based on analysis of the partial 28S rDNA sequences, the species is most closely related to those in the Waretrematinae with a single caecum.

Introduction

The collection of squaretail mullet from Australia yielded specimens of an unknown genus and species of Haploporidae Nicoll, 1914. The new genus and species are described below, and their position in the Haploporidae is discussed.

Materials and Methods

Fish hosts were collected by castnet. Identification of the host conforms to currently valid names and common name given in FishBase (Pauly & Froese, 2013). Haploporids were isolated similarly to the process advocated by Cribb and Bray (2010)

for gastrointestinal species, skipping an initial examination because of the volume of the intestinal contents. Live worms were rinsed and cleaned in saline and observed briefly. The saline was removed from the container, and the worms were killed by addition of hot steaming, but not boiling, tap water. Haploporids were then stored in 70% ethanol. Parasite specimens for morphological and molecular analysis were processed and studied according to the protocols used by Pulis et al. (2013) and Pulis and Overstreet (2013). All measurements are in micrometres unless noted otherwise. Museum abbreviations are as follows: MNT, Museum and Art Gallery of the Northern Territory, Darwin, Australia; and USNPC, US National Parasite Collection, Beltsville, Maryland.

Genomic DNA was isolated using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the instructions provided. DNA fragments approximately 2,500 basepairs (bp) long comprising the 3' end of the 18S nuclear rDNA gene, internal transcribed spacer region (including ITS1 + 5.8S + ITS2), and the 5' end of the 28S 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 primers ITSF (5' - CGCCCGTCGCTACTACCGATTG-3') or LSU5 (5'-TAGGTCGACCCGCTGAAYTTAAGCA-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'), 900F (5'-CCGTCTTGAAACACGGACCAAG-3'), and internal reverse primers were 300R (5'-CAACTTTCCCTCACGGTACTTG-3'), Digl2r (5'-CCGCTTAGTGATATGCTT-3'), and ECD2 (5'-CTTGGTCCGTGTTTCAAGACGGG-

3’). Previously published 28S ribosomal RNA gene sequences of species of Haploporidae were used for comparison (see Figure 11 for accession numbers) with newly submitted sequences. Sequences were aligned using the ClustalW application in the BioEdit program, Version 7.0.9 (Hall, 1999). The alignment was further refined by eye and trimmed to the shortest sequence on both 5' and 3' ends. The resulting alignment utilised 32 haploporids with *Hapladena nasonis* as the outgroup, and the alignment was 1,098 characters long, including gaps, with 727 sites conserved, 371 sites variable, and 279 parsimony-informative sites. Phylogenetic analysis of the data was performed using Bayesian Inference (BI) with MrBayes 3.1.2 software (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The best nucleotide substitution model was estimated with jModeltest Version 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) as general time reversible with gamma-distributed among site-rate variation and estimates of invariant sites (GTR + I + Γ). The following model parameters were used in MrBayes: nst = *6*, rates = *invgamma*, ngen = *1,000,000*, and samplefreq = *100*. The first one quarter of samples were discarded as burn-in (*sump burnin = 2500*), and nodal support was estimated by posterior probabilities (*sumt*) (Huelsenbeck et al., 2001). All other settings were left as default.

Results

New genus 1

Diagnosis: Body of adult elongate, approximately 5 times longer than wide, with posterior end tapered. Eye-spot pigment dispersed. Tegument spined. Oral sucker subterminal, opening to mouth with anterior cleft, single row of small spines along posterior ventral margin ventral to mouth. Ventral sucker prior to equatorial. Prepharynx

long. Pharynx longer than wide. Caecum near mid-hindbody, bilobed, wider than long. Testis in hindbody near posterior margin of ventral sucker. Hermaphroditic sac dorsal to ventral sucker, elongate, thick-walled, and originating noticeably posterior to genital pore. Hermaphroditic duct about 1/3 the length of hermaphroditic sac, muscular, with smooth appearance. Male duct short. Female duct long, muscular, rugose in appearance. Internal seminal vesicle well-defined, straight, not sac-like. External seminal vesicle round, made up of large nucleated cells. Ovary contiguous with anterior portion of testis. Uterus long, convoluted, extending posterior to testis, metraterm absent. Vitellarium dorsal to ovary and testis, composed of about 15 lobes. Eggs thin-shelled, distal eggs containing developed miracidium with eye-spots. Excretory vesicle I-shaped with two collecting tubes originating from anterior portion. Excretory pore subterminal dorsal. Lymphatic system present. In intestine of Mugilidae from Indo-West Pacific region. Type-species: New genus 1 new species.

Remarks

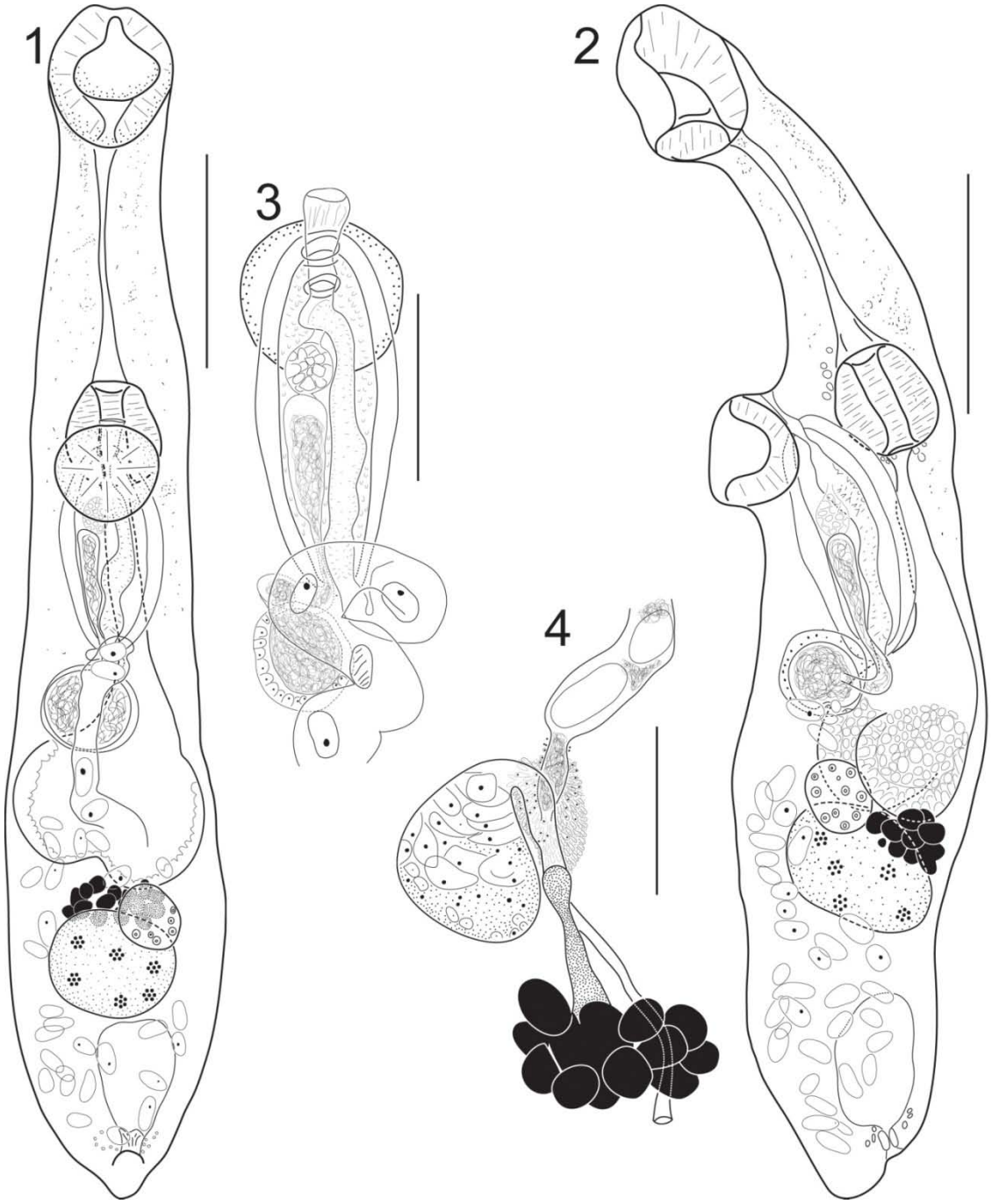
In its possession of a spined tegument, hermaphroditic sac, and single testis, N. g. 1 is a member of the family Haploporidae Nicoll, 1914.

New genus 1 new species can be separated from other genera in the family Haploporidae by the combination of single caecum, reduced vitellarium, uterus extending into post-testicular area, in-utero eggs containing developed miracidium with an eye-spot, I-shaped excretory vesicle, and sub-terminal dorsal excretory pore. Additional characters unique to N. g. 1 n. sp. include oral sucker with anteroventral cleft; oral sucker armed posteriorly with single row of small spines; caecum wider than long; hermaphroditic sac muscular, thick walled; non-sac-like internal seminal vesicle; and external seminal

vesicle lined with large nucleated cells. The gross anatomy of N. g. 1 n. sp. is most similar to the genus *Unisaccus* Martin, 1973 due to the single caecum, reduced vitellarium, uterus extending post-testicularly, and in-utero eggs containing miracidium with eye spots. Characters possessed by *Unisaccus* not found in N. g. 1 n. sp. include spirally arranged pads in the posterior end of the hermaphroditic duct, sac-like internal seminal vesicle, sac-like external seminal vesicle, and Y-shaped excretory vesicle. The presence of eye-spots in the miracidium of in-utero eggs distinguishes N. g. 1 n. sp. from *Unisaccoides* Martin, 1973 and *Paraunisaccoides* Martin, 1973. *Unicoelium* Thatcher & Dossman also has a single caecum as found in N. g. 1 n. sp., but possesses vitellarium in two distinct clumps lateral to ovary, uterus extending anterior to hermaphroditic sac, and Y-shaped excretory vesicle. *Pseudolecithobotrys* Blasco-Costa, Gibson, Balbuena, Raga, & Kostadinova, 2009 has a variable caecal shape ranging from singular to bifurcating and differs from N. g. 1 n. sp. by short prepharynx, the sac-like external seminal vesicle, muscular genital atrium, and vitellarium in two distinct clumps.

New genus 1 new species (Figures 1-11)

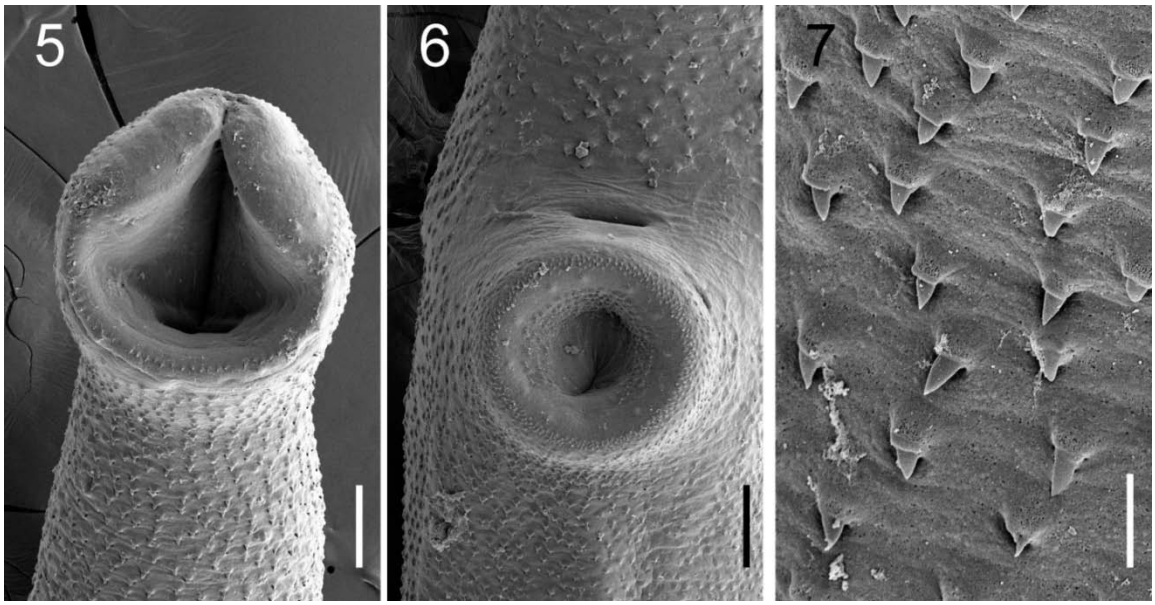
Description: Measurements based on 15 wholemount specimens. Body elongate, 1552-2016 long (BL), 254-396 wide, widest in hindbody, width 16-22% of BL. Tegument spined; spines hasate, about 10-12 long in forebody, majority of spine beneath surface of tegument (Figure 7) becoming smaller and more sparse posteriorly. Eye-spot pigment dispersed, most prominent in forebody. Oral sucker subterminal, anterior half cleft (Figures 1, 5), 174-232 long, 168-221 wide, with mouth opening anteroventrally, posterior ventral margin or oral sucker with single row of minute spines (Figure 5).



Figures VI.1-4. New genus 1 new species 1. Holotype ventral wholemount; 2. Paratype lateral whole mount; 3. Paratype hermaphroditic sac, ventral mount ventral sucker outline shown; 4.Paratype ovarian complex. S*cale-bars* 1-2. 300µm, 3. 200µm, 4. 100 µm.

Ventral sucker slightly elevated, 134-180 long, 138-188 wide, with about four rows of small spines on most ventral rim of ventral sucker, inner circle of 12 papilla, inside of papilla a few rows of spines smaller then body spines, larger than three rows of the rim, deepest portion unarmed (Figure 6). Ventral sucker 68-87% as long as oral sucker, 73-88% as wide as oral sucker. Forebody 500-762 long or 34-40% of BL; hindbody 848-1222 long or 52-62% of BL. Prepharynx well defined, 274-420 long. Pharynx 140-184 long, 123-167 wide, length 102-125% of width, widest in posterior half. Oesophagus 313-577 long. Caecum bilobed, 150-254 long, 218-326 wide, lined with large cells (which was used to determine the anterior extent), commencing 931-1322 from anterior end of body or 58-69% of BL; post-caecal space 344-637 or 18-32% of BL.

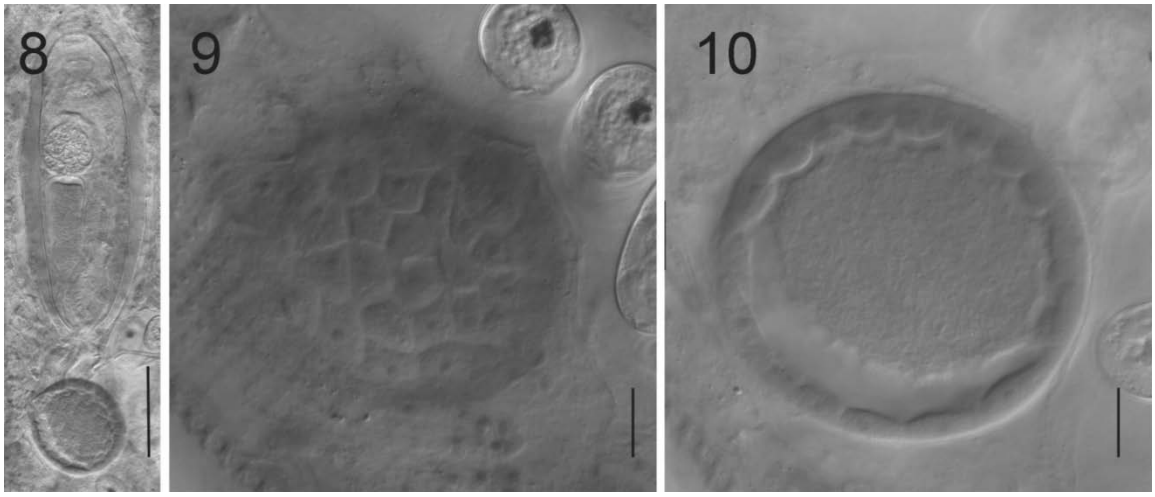
Testis medial, round, overlapping posterior end of caeca to 92 posterior, 362-670 from ventral sucker, 132-171 long, 140-203 wide; post-testicular space 220-449 or 14-24% of BL. Hermaphroditic sac dorsal to ventral sucker, 301-469 long, 122-160 wide, outer muscular wall about 20 thick, terminating posterior to ventral sucker; sac containing following structures (internal seminal vesicle straight, well-defined, 109-180 long, 24-50 wide; male duct thin-walled; prostatic bulb immediately anterior to internal seminal vesicle; female duct muscular, appearance rugose; male duct joining female in anterior third of sac); hermaphroditic duct smooth. External seminal vesicle 107-164 long, 90-162 wide, with definite layer of thick nucleated cells, about 10-15 thick. Genital pore laterally elongate, immediately anterior to ventral sucker, hermaphroditic duct



Figures VI.5-7. New genus 1 new species scanning electron micrographs. 5. Oral sucker showing anterior cleft and row of small spines on posterior ventral rim; 6. Ventral sucker with papillae and laterally elongate genital pore; 7 Tegumental spines of forebody. *Scale-bars: 5. 50µm; 6. 50µm; 7. 10µm.*

noticeably long from hermaphroditic sac to genital pore, area immediately around genital pore devoid of spines.

Ovary medial, contiguous with testis, ventral to testis, 63-101 long, 67-110 wide. Mehlis' gland antero-dorsal to ovary. Laurer's canal dorsal to testis; pore dorsal to testis. Vitellarium consisting of mass of about 9-20 lobes (number subjective), nestled dorsal to ovary, between testis and caecum. Uterus convoluted, occupying most of hindbody posterior to hermaphroditic sac; proximal portion often filled with sperm; metraterm absent. Eggs thin-shelled, yellow, numerous, 50-67 long, 22-38 wide (measured from fully developed distal portion of uterus), considerable variation in size between specimens (weak correlation of longer worm = longer egg), with developed miracidium possessing eye-spot.



Figures VI.8-10. New genus 1 new species micropictographs. 8. hermaphroditic sac showing thick muscular wall; 9. external seminal vesicle, ventral surface showing large nucleated cells; 10. external seminal vesicle mid-depth showing thickness of nucleated cells. *Scale-bars*: 8. 100µm; 9. 20µm; 10. 20µm.

Lymphatic tubes in forebody. Excretory vesicle I-shaped, posterior to testis, with two narrow cura emerging from anterior portion; pore dorsal subterminal.

Type-host: *Ellochelon vaigiensis* (Quoy & Gaimard), squaretail mullet (Mugilidae)

Type-locality: Ludmilla Creek, Darwin, Northern Territory, Australia (caught on mud flat that appeared to be flooded only during highest spring tides). (12° 24' 52" S, 130° 50' 10" E).

Additional locality: Whithnell Bay, Burrup Peninsula, near Dampier, Western Australia, Australia, (20° 35' 01" S, 116° 47' 21" E).

Site: Intestine.

Type-material: MNT TBD holotype TBD paratype; BM TBD paratypes; USNPC TBD Paratypes. Gen bank TBD

Remarks

New genus 1 new species is the only species within the genus.

Phylogenetic Analysis

In the BI analysis of partial 28S sequences presented here N. g. 1 n. sp. was found to be within the clade considered Waretrematinae by Pulis and Overstreet (2013), sister to the clade of other single caecum waretrematins (*Malabarotrema* Zhukov, 1972; *Unisaccoides* Martin, 1973; *Unisaccus* Martin, 1973) (Figure 11)

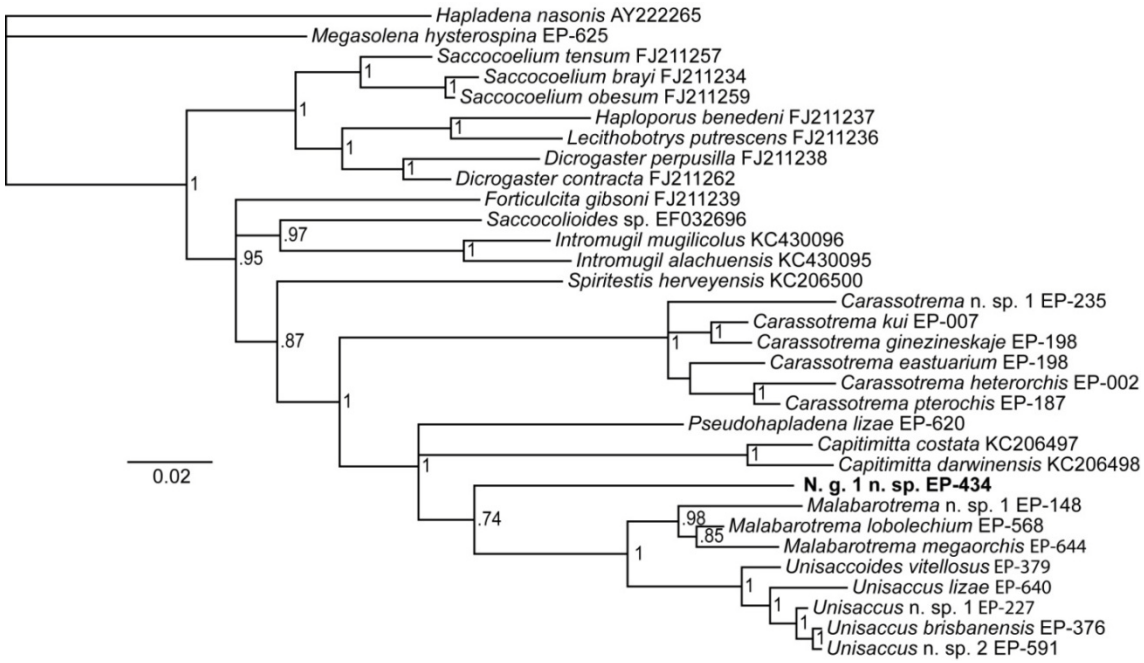


Figure VI.11. Estimated position of New genus 1 new species within the Haploporidae. Bayesian Inference tree of phylogenetic relationships of the Haploporidae using partial 28S rDNA sequences. Position of New genus 1 new species shown in bold.

Discussion

Molecular analysis of partial 28S rDNA fragments of haploporid genera with N. g. 1 n. sp. reveal that is not particularly related to any genus from which sequence data is available. Based on the most recent comprehensive evaluation of the family by Overstreet and Curran (2005), I would have expected N. g. 1 n. sp. to be closer to the Haploporinae

than to the Waretrematinae. In the assessment of some species possessing a single caecum in the previous chapter, *Unisaccus* was found to be a member of the Waretrematinae, which was not suspected by previous authors who speculated on the subfamilial relations of the family. The defining characters for the Haploporinae were proposed as the in-utero eggs with developed miracidium possessing eye-spots and reduced vitellarium. Now that another genus with eye-spotted miracidium, reduced vitellarium, and a single caecum has been proposed in this paper it beguiles attempts to form a morphological basis for the subfamilies as recognized by Overstreet and Curran (2005), although as shown by other phylogenetic work on the family (Blasco-Costa et al, 2010; Pulis & Overstreet, 2013) there are clear clades that I believe merit subfamily designations. After the work of Blasco-Costa, Balbuena et al. (2009) phylogenetic analysis of the family has been limited to the addition of species that happen to fall within the Waretrematinae clade, while no species have been added from the Haploporinae or Forticulcitanae Blasco-Costa et al. (2010) clades. I do not expect the addition of more species to the tree produced here to change the conclusions of this paper, but additional species will provide more robust inferences to the stability and usefulness of characters to determine relationships.

New genus 1 new species has several characters not reported for any other haploporid genera, including armed posterior oral sucker, papilla encircling the ventral sucker, and the robust external seminal vesicle composed of large nucleated cells. Also, the internal seminal vesicle is more robust than has been reported for other members of the family, but many original and subsequent descriptions have been based on material killed under coverslip pressure, which lessens the utility of this character. Because N. g. 1

n. sp. possesses many unusual characters compared with other described species, I consider these features to be derived.

The present study demonstrates that morphological characters used by Overstreet and Curran (2005) to differentiate the Waretrematinae from Haploporinae are not of systematic importance. Based on 28S partial sequence analyses (Figure 11) Haploporinae and Waretrematinae form well-supported, distinct clades. The extensive uterus, reduced vitellarium, and well-developed miracidium in-utero are all characters that are found in other subfamilies of haploporid other than Megasoleninae, species which occupy a basal position in the Haploporidae. This suggests that reduced vitellarium and well-developed miracidium in-utero have been acquired at least a few times in the family. *Ellochelon vaigiensis* that were collected for this work were actively feeding in waters at the highest spring tides (personal observation). This along with the advanced development of the miracidium in-utero could suggest that the miracidium leaves the fish and seeks out a snail host, whereas an egg that requires and incubation period outside the host may be subject to desiccation. I suspect that the first intermediate host of N. g. 1 n. sp. is a intertidal snail.

CHAPTER VII

NEW GENUS 2 NEW SPECIES FROM THE CLUPEID *NEMATALOSA COME* FROM AUSTRALIA

Abstract

New genus 2 is proposed for New genus 2 new species collected from the clupeid *Nematalosa come*. The new genus is most similar to the genera *Carassotrema* and *Platydidymus.* New genus 2 can be distinguished from *Platydidymus* by the lack of a crenulated oesophagus, Y-shaped rather than H-shaped gut, and vitellarium not extending anterior to testis. While two species of *Carassotrema* have been reported from Clupeids, an unusual host for haploporids, N. g. 2 can be differentiated from *Carassotrema* species by the vitellarium being a mass of follicles not organized into distinct tubes or plates restricted dorsally and posterior to testis but not anterior, and gut being sac-like rather than tubular. Based on a 28S rDNA gene fragment Bayesian Inference analysis suggest that N. g. 2 n. sp. is most closely related to species of the genus *Carassotrema*.

Introduction

Collections of *Nematalosa come* (Richardson, 1846) from Australia were infected with specimens of a member of the family Haploporidae Nicoll, 1914 that are not attributable to any known genus. Therefore, I propose a new genus for the new species.

Materials and Methods

Fish hosts were collected by castnet. Names of fish host conforms to currently valid names and common name given in FishBase (Froese & Pauly, 2013). Haploporids were isolated as advocated by Cribb and Bray (2010) for gastrointestinal species. Live worms were rinsed and cleaned in saline and observed briefly. The saline was removed

from the container, and the worms were killed by addition of hot, but not boiling, tap water. Haploporids were then stored in 70% ethanol. Parasite specimens for morphological and molecular analysis were processed and studied according to the protocols used by Pulis et al. (2013) and Pulis and Overstreet (2013). All measurements are in micrometres unless otherwise noted. Museum abbreviations are as follows: MNT, Museum and Art Gallery of the Northern Territory, Darwin, Australia and USNPC, US National Parasite Collection, Beltsville, Maryland.

Genomic DNA was isolated using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the instructions provided. DNA fragments approximately 2,500 basepairs (bp) long comprising the 3' end of the 18S nuclear rDNA gene, internal transcribed spacer region (including ITS1 + 5.8S + ITS2), and the 5' end of the 28S 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 primers ITSF (5' - CGCCCGTCGCTACTACCGATTG-3') or LSU5 (5'-TAGGTCGACCCGCTGAAYTTAAGCA-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’), 900F (5’-CCGTCTTGAAACACGGACCAAG-3’), and internal reverse primers were 300R (5’-CAACTTTCCCTCACGGTACTTG-3’), Digl2r (5’-CCGCTTAGTGATATGCTT-3’), and ECD2 (5’-CTTGGTCCGTGTTTCAAGACGGG-3’). Previously published 28S ribosomal RNA gene sequences of species of Haploporidae were used for comparison (see Figure 11 for accession numbers) with newly submitted

sequences. Sequences were aligned using the ClustalW application in the BioEdit program, Version 7.0.9 (Hall, 1999). The alignment was further refined by eye and trimmed to the shortest sequence on both 5' and 3' ends. The resulting alignment utilised 32 haploporids with *Hapladena nasonis* as the outgroup, and the alignment was 1,098 characters long, including gaps, with 727 sites conserved, 371 sites variable, and 279 parsimony-informative sites. Phylogenetic analysis of the data was performed using Bayesian Inference (BI) with MrBayes 3.1.2 software (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The best nucleotide substitution model was estimated with jModeltest Version 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) as general time reversible with gamma-distributed among site-rate variation and estimates of invariant sites (GTR + I + Γ). The following model parameters were used in MrBayes: nst = *6*, rates = *invgamma*, ngen = *1,000,000*, and samplefreq = *100*. The first one quarter of samples were discarded as burn-in (*sump burnin = 2500*), and nodal support was estimated by posterior probabilities (*sumt*) (Huelsenbeck et al., 2001) with all other settings left as default.

Results

New genus 2 Figures. 1-3.

Diagnosis: Body minute, pyriform. Tegument spined. Diffuse eye-spot pigment prominent in forebody. Oral sucker terminal. Ventral sucker near equatorial. Prepharynx long. Pharynx smaller than suckers. Oeseophagus smooth, long, near length of prepharynx, terminating near posterior margin of ventral sucker. Caeca sac-like, in hindbody, reaching posterior to testis. Testis large, V-shaped, wider than long.

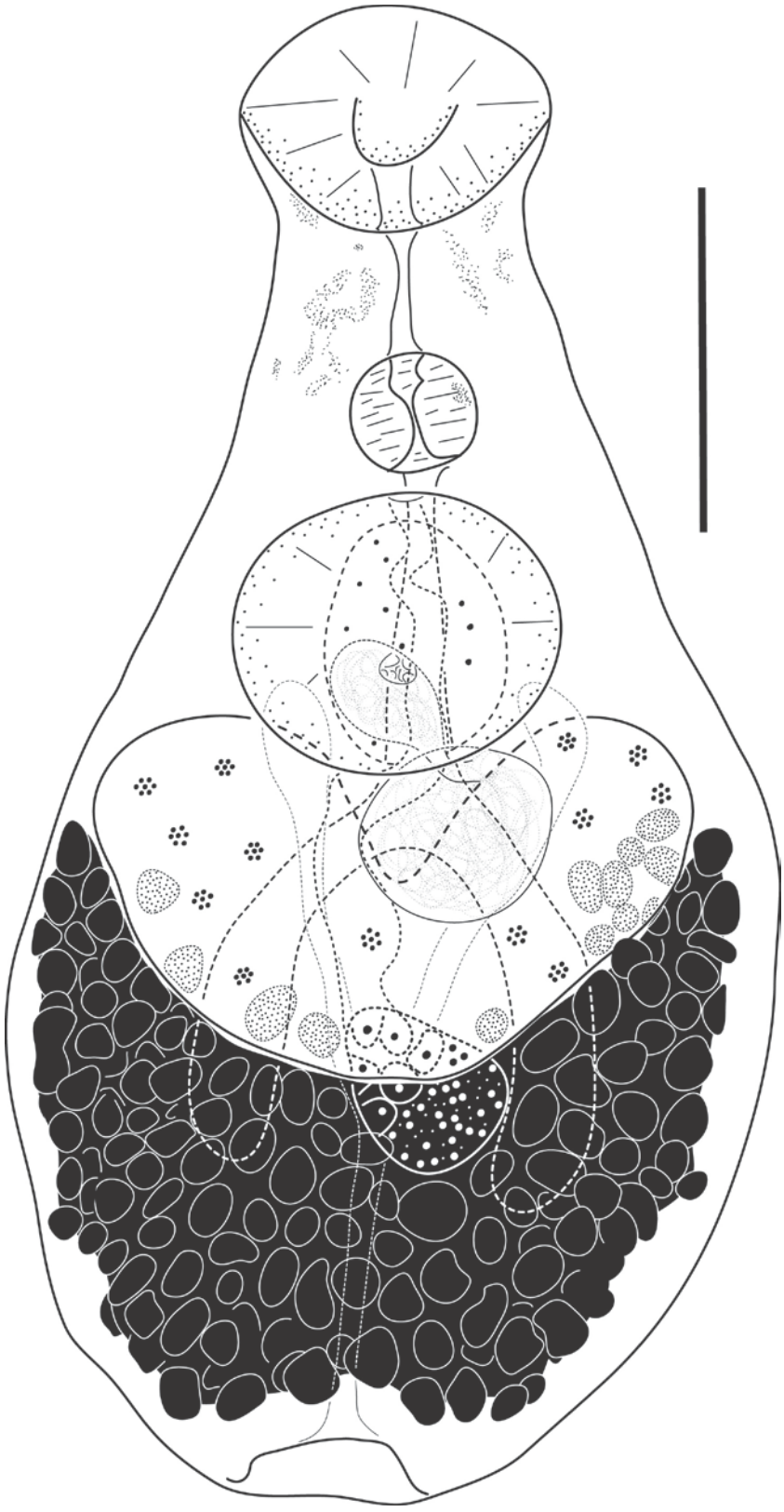


Figure VII.1. New genus 2. 1.Ventral wholemount *scale-bar* 100 µm.

Hermaphroditic sac ellipsoidal. Internal seminal vesicle occupying less than half of hermaphroditic sac. External seminal vesicle sac-like, roughly same size as internal seminal vesicle. Ovary round, dorsal to testis. Uterus direct from ovary to hermaphroditic sac, proximal portion filled with sperm. Vitellarium of homogenous mass of rounded follicles surrounding posterior and dorsal portion of testis to posterior end of body. Eggs not observed. Excretory vesicle Y-shaped; pore terminal. In Clupeidae Indo-West Pacific region. Type-species: N. g. 2. n. sp.

Remarks

New genus 2 possess a spined tegument, a single testis, and a hermaphroditic sac consistent with other members of the family Haploporidae Nicoll, 1914. New genus 2 can be differentiated from other genera in the family by the combination of characters, including pyriform body, V-shaped, wide testis, ovary dorsal to testis, and vitellarium confined to area posterior to posterior margin of testis.

New genus 2 is most similar to the genera *Carassotrema* Park, 1938 and *Platydidymus* Overstreet and Curran, 2005 (syn. *Haplotrema* Zhukov, 1971 preoccupied). New genus 2 can be differentiated from *Carassotrema* by the vitellarium not extending anterior to testis and from *Platydidymus* by the sac-like non H-shaped gut configuration, smooth oesophagus, and vitellarium not extending anterior to testis.

Specimens for members of this genus were collected from *Nematlosa come* from Western Australia and Queensland, Australia. Due to the lack of molecular material from the Queensland specimen, I only include specimens from Western Australia in the description of N. g. 2 n. sp. The specimen from Queensland, Australia is certainly congeneric with N. g. 2 n. sp. from Western Australia, but it may prove to be separate

species once the eastern form has more specimens collected. Due to the lack of molecular data from the eastern specimen I take a very conservative approach an decline to include it in the description of the new species, although it was used in the generic diagnosis.

New genus 2 new species (Figures 2-3)

Type-host: *Nemataslosa come* (Richardson), western Pacific gizzard shad (Clupeidae).

Site: Intestine.

Type-locality: Barred creek, Western Australia, Australia (17° 39' 48" S 122° 12' 06" E).

Description: Measurments based on 2 wholemount worms. Body pyriform, 285-333 long, 134-162 wide, measured in hindbody where it is greatest. Body width 47-49% of length. Tegument armed with spines about 4 long, becoming smaller and more sparse toward the posterior end of the body, small size of the body gives anterior end thorny appearance. Oral sucker sub-terminal, 52-63 long, 64-76 wide. Ventral sucker slightly elevated, pad-like, without deep depression in center, 64-75 long, 74-83 wide, 119-123% longer than oral sucker, 109-116% wider than oral sucker. Forebody 104-114 long or 34-36% of BL; hindbody 117-151 long or 41% of BL. Prepharynx 20-25 long. Pharynx muscular 31-37 long, 29-36 wide, length 124-185% of prepharynx length. Oesophagus 47-58 long, bifurcating near posterior margin of ventral sucker, 154-180 from anterior end of body, 54% of body length. Caeca long and tubular, ending blindly 62-64 from posterior end of body; postcaecal space 21-24% of BL.

Testis medial, strongly V-shaped, orientated nearly vertically with point of V dorsal, with tips of V near ventral surface, 68-71 long, 100-122 wide, width 147-172% of

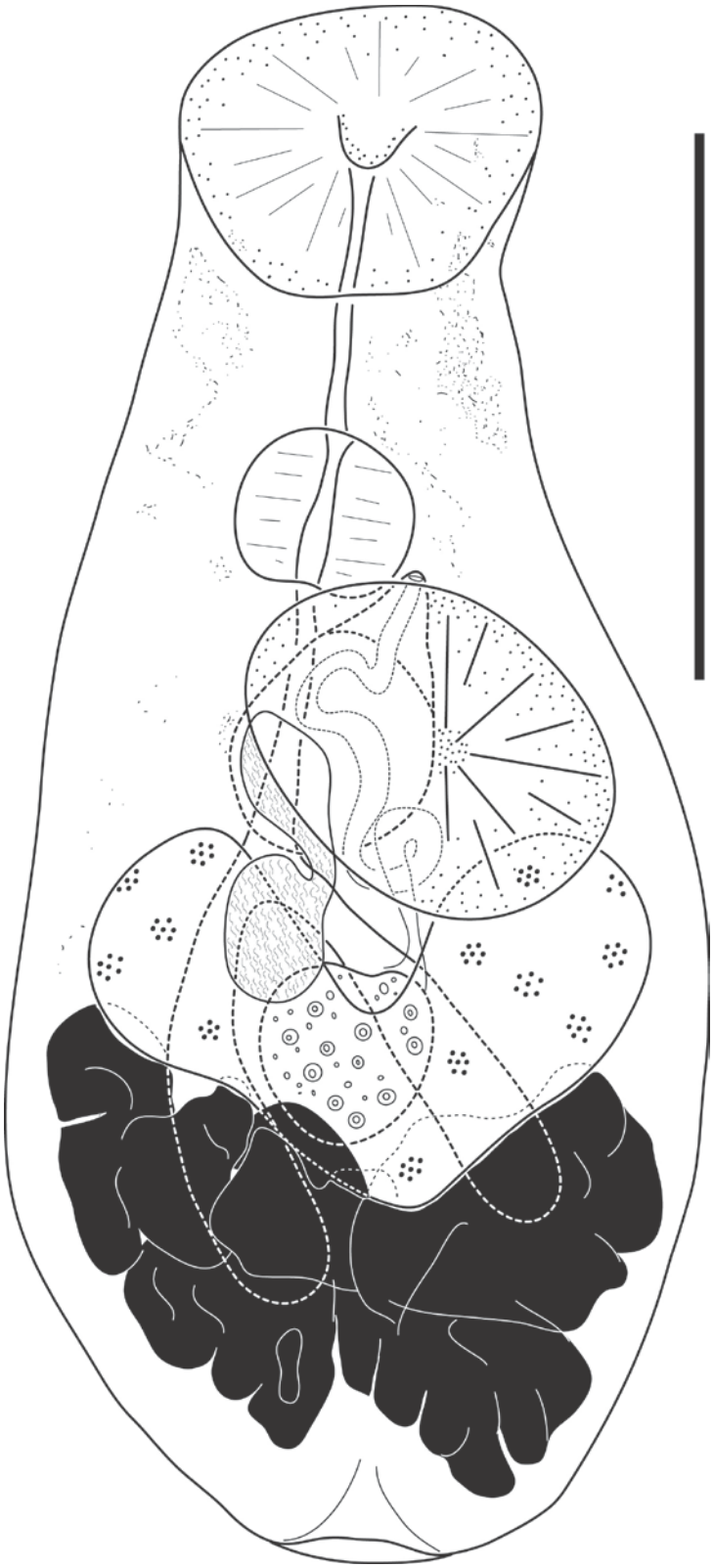


Figure VII.2. New genus 2 new species 2. ventral wholemount Holotype. S*cale-bar*, 100µm.

length, anterior tips at level of posterior quarter of ventral sucker, 52-92 from posterior end, post-testicular space 18-28% of BL. Hermaphroditic sac, medial, dorsal to ventral sucker, 45-61 long, 26-37 wide, containing internal seminal vesicle, 22-25 long, 16-17 wide, male duct not observed, female duct smooth, and hermaphroditic duct proximal end muscular and smooth distal end smooth. Genital pore medial, immediately at anterior margin of ventral sucker. External seminal vesicle sac-like, 23-51 long, 21-23 wide.

Ovary medial, dorsal to midpoint of testis, 30-42 long, 31-38 wide, 12-14 posterior to ventral sucker. Ootype surrounded by Mehlis' gland, situated anterior to ovary. Laurer's canal not observed. Vitellarium extensive, follicles in plate-like sections, commencing near level of posterior half of testis, filling most of post-testicular space. Uterus occupying space between ovary and hermaphroditic sac, proximal end filled with sperm, distal end without evidence of metraterm. Eggs not observed. Excretory bladder Y-shaped, bifurcation dorsal to posterior end of testis; pore terminal.

Molecular Results:

N. g. 2. n. sp. shows the greatest affinity to Species of *Carassotrema* according to the BI analysis (Figure 3).

Discussion

Clupeids are an overlooked host for haploporids. To date only three species of haploporids have been recorded from the family, two in *Carassotrema* (Liu, 2003; Chapter V) and the new genus reported here. Further sampling of Indo-Pacific clupeids should yield more undescribed species. The subfamily Waretrematinae Srivastava, 1939, of which N. g. 2 is a member, is hosted predominantly by mullets. The

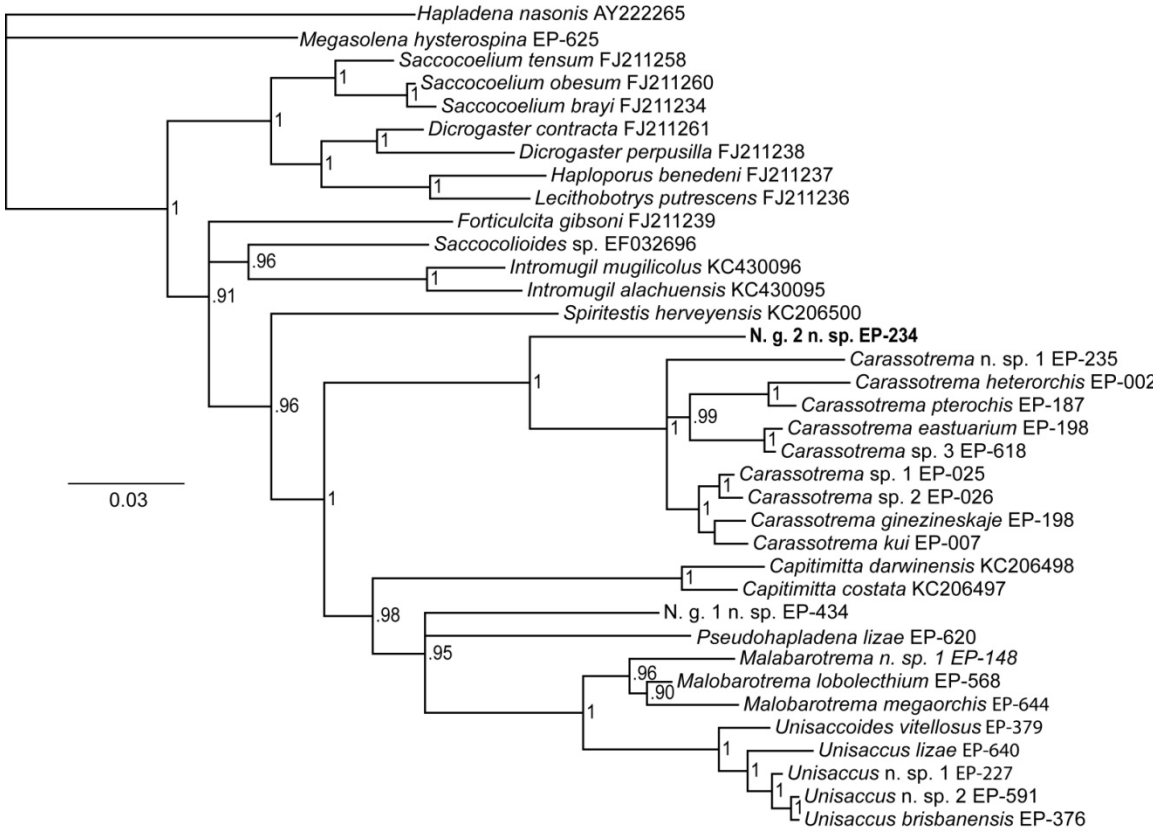


Figure VII.3. Estimated position of New Genus 2 New species 3. Bayesian Inference tree of phylogenetic relationships of the Haploporidae using partial 28S rDNA sequences with *Hapladena nasonsis* as the outgroup. N. g.2 n. sp. shown in bold. Posterior probability scores greater than 0.80 given.

finding of representatives of two genera in these fishes is intriguing. The known life cycles of haploporids involve two hosts with the cercaria or metacercaria being ingested by the definitive host (Martin, 1973a; Tang & Lin, 1979; Shameen & Madhavi, 1991; Yu et al., 2005). As such, those clupeids which are detritivores, like mullets, should provide ample opportunity for host switching, indicating the host associations in the haploporids are the result of host switching rather than co-evolution.

Although eggs were not observed, I suspect that they would not contain a developed miracidium with eye-spots while in the uterus. Those members of the waretrematins, *Unisaccus* and N. g. 1, have in-utero eggs with developed miracidium

with eye-spots, have a long uterus and reduced vitellarium, whereas N. g. 2 has a short uterus and extensive vitellarium. I predict that, when observed, the eggs of N. g. 2 when deposited will lack developed miracidium and take some time to mature before being infective to the first intermediate host as in the two species of *Carassotrema* with known life-cycles (Tang & Lin, 1979).

CHAPTER VIII

CHANGE IN RANK OF MEGAPERIDAE (TREMATODA) TO THE MEGAPERINAE WITHIN THE APOCREADIIDAE AND DESCRIPTION OF *HAINTESTINUM AMPLUM* N. G. N. SP.

Abstract

Haintestinum amplum n. g. n. sp. is described from the scrawled cowfish, *Acanthostracion quadricornis*, collected in the Eastern Gulf of Mexico off Florida, USA. The new species is relatively large and shares characters of the Apocreadiidae and Megaperidae but conforms to the diagnosis of neither. It belongs in a new genus possessing a pharynx with lobed anterior margin and intestine terminating in paired ani, like in megaperids, and, when compared with apocreadiids, it shares important anatomical features, including an I-shaped excretory vesicle, canicular seminal vesicle, eye-spot remnants, and pretesticular uterus and lacks a cirrus and cirrus sac. The H-shaped intestine and large funnel-shaped oral sucker without a U-shaped sphincter encircling half the anterior aperture are the most notable diagnostic characters of the new monotypic genus. Additionally, the phylogenetic position of the Megaperidae is investigated for the first time, using analysis of partial 28S rDNA gene sequences from *H. amplum*, two species in the *Megapera*, *Thysanopharynx elongatus*, and previously published 28S sequences of species from members of the Apocreadiata, Haploporoidae, Lepocreadiata, and Opisthorchiata. The resulting analysis demonstrated a close relationship among the new genus and the three species of megaperids, and the megaperids were most closely allied with *Schistorchis zancli* of the apocreadiids.

Moreover, I now consider Megaperidae as the subfamily Megaperinae within the Apocreadiidae.

Introduction

Prior to the present study, five species in the genus *Megapera* Manter, 1934 and *Thysanopharynx elongatus* Manter, 1933 comprised the Megaperindae Manter, 1934, all of which are parasites of tetradontiform fishes from the warm waters of the western North Atlantic Ocean. Manter (1933) initially noted that adult megaperids shared similar traits with worms that were considered to belong in the Allocreadiidae Looss, 1902 and the Lepocreadiidae Odhner, 1905 at that time, but Manter deemed that the presence of paired ani, an undivided seminal vesicle, pre-ovarian symmetrical testes situated in the forebody, and peculiar shaped pharynx plus the lack of a cirrus and cirrus sac served to unite the group as a family. Cable (1954) investigated the life history of *Megapera gyrina* (Linton, 1907) Manter, 1934, and observed that the cercaria emerged from a slipper limpet (*Crepidula convexa* Say, 1822) at Salinas Bay, Puerto Rico, USA, and encysted almost immediately on vegetation or substratum, suggesting lack of a second intermediate host, and developed in a herbivorous definitive host that fed on infested vegetation.

Manter (1963) reassessed the relationship between the Megaperidae and the Lepocreadiidae Odhner, 1905, noting similarities between specialization of the oral sucker in the megaperids and certain genera that were initially considered to belong in the Lepocreadiidae, but presently considered in the Apocreadiidae Skrjabin, 1942 (i.e., *Schistorchis* Lühe, 1906, *Sphincterostoma* Yamaguti, 1937, *Megacreadium* Nagaty, 1956 and *Lobatotrema* Manter, 1963 [syn. *Sphincteristomum* Oshmarin, Mamaev, and

Parukhin, 1961]). Manter (1963) reduced the family Megaperidae to the subfamily rank Megaperinae Manter, 1963. *Megapera* and *Thysanopharynx* Manter, 1933 had previously considered to be in the Lepocreadiidae; *Sphincterostoma* and *Sphincteristomum* were considered in the Sphincterostomatinae Yamaguti, 1958; and *Megacreadium* was considered a synonym of *Schistorchis* in Lepocreadiidae without placement in a subfamily (Manter, 1963; Sogandares-Bernal & Hutton, 1959). Yamaguti (1971) transferred *Schistorchis* and *Megacreadium* to the family Schistorchiidae Yamaguti, 1942 and treated the two megaperid genera in their own family, Megaperidae, based on morphological features of the cercaria of *M. gyrina*. Mehra (1961) and Brooks et al. (1985) also questioned an affinity between the Megaperinae and the Lepocreadiidae based on the presence of a Y-shaped excretory bladder in the cercaria of *M. gyrina*, suggesting a relationship with the Haploporidae Nicoll, 1914. Cribb and Bray (1999) proposed that *Schistorchis*, *Sphincterostoma*, *Megacreadium*, and *Sphincteristomum* all belonged in the subfamily Schistorchiinae Yamaguti, 1942 in the Apocreadiidae. In the most recent classification of the Trematoda, Bray (2005a; 2005b) tentatively treated the Megaperidae as a family within the artificial Superfamily Lepocreadioidea Odhner, 1905. In the most recent molecular phylogeny (Bray et al., 2009) and treatment (Bray & Cribb, 2012) of the superfamily Lepocreadioidea, three families, Megaperidae, Liliatrematidae Gubanov, 1953, and Deropristidae Cable and Hunninen, 1942, have not had molecular data available to support or challenge their inclusion into that or another superfamily.

In the present study, I present a description of a new species and genus that possesses morphological features that are diagnostic for both the Apocreadiidae and the Megaperidae, provide sequence data for the new genus and three species of megaperids,

and conduct an analysis of sequence data estimating the phylogenetic relationships of the new species and the megaperids.

Materials and Methods

The scrawled cowfish, *Acanthostracion quadricornis* (Linnaeus), and honeycomb cowfish, *Acanthostracion polygonius* Poey were collected during the National Marine Fisheries Service (NMFS) Fall Pelagic Trawls in the eastern Gulf of Mexico (GOM) from 2007 through 2010 and by cast net off Grassy Key, Florida, in 2012. Fish were examined for helminths while fresh. The trematodes for this study were cleaned in saline, examined briefly under a dissecting scope, killed by submersion in hot water, and fixed in 70% ethanol for specimens used in molecular analyses, morphological identifications, and scanning electron microscopy (SEM). Specimens used for wholemounts were stained in aqueous Mayer's hematoxylin or Van Cleave's hematoxylin, dehydrated in a graded ethanol series, cleared in clove oil (Van Cleave's) or methyl salicylate (Mayer's), and mounted permanently in Canada balsam. Measurements were taken with a differential interference contrast (DIC) equipped Leica compound microscope using a ProgRes® CapturePro camera (Version 2.8 Jenoptic, Jena, Germany) and software. All measurements are in micrometres (µm) unless noted otherwise. Sequences used in the present study were either derived from trematode specimens I collected from the intestines of fishes between 2007 and 2012 (Table I) or obtained from GenBank. The type specimens of the new species and representative voucher specimens were deposited at the United States National Parasite Collection (USNPC), Beltsville, Maryland.

Genomic DNA was extracted from two hologenophores (terminology of Pleijel et al., 2008) of the new species, and one hologenophore of *T. elongatus*, and entire single

specimens of *M. gyrina* and *Megapera orbicularis* (Manter, 1933), using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the instructions provided. DNA fragments measuring approximately 2,500 basepairs (bp) long comprising the 3' end of the 18S nuclear rDNA gene, internal transcribed spacer region (including ITS1 + 5.8S + ITS2), and 5' end of the 28S gene (including variable domains D1-D3) were amplified from the extracted DNA by polymerase chain reaction (PCR) using a PTC-200 Peltier Thermal Cycler and forward primers ITSF (5' - CGCCCGTCGCTACTACCGATTG-3') or LSU5 (5'-TAGGTCGACCCGCTGAAYTTAAGCA-3') and reverse primer 1500R (5'-GCTATCCTGAGGGAAACTTCG-3'). These PCR primers and additional internal primers were used in sequencing reactions. Those additional internal forward primers were Digl2 (5'-AAGCATATCACTAAGCGG-3'), 300F (5'-AAGTACCGTGAGGGAAAGTTG-3'), and 900F (5'-CCGTCTTGAAACACGGACCAAG-3'), and internal reverse primers were 300R (5'-CAACTTTCCCTCACGGTACTTG-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 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 were assembled using Sequencher™ (GeneCodes Corp., Ann Arbor, Michigan, USA, Version 5.0), and representative sequences were submitted to GenBank.

Newly obtained sequences (Table 1) and sequences of purported relatives of the Megaperidae were used in the phylogenetic analysis. Sequences obtained from GenBank and used in the present analysis are listed as follows: *Caecincola parvulus* Marshall and Gilbert, 1905 AY222231, *Adlardia novaecaledoniae* Miller, Bray, Goiran, Justine, and Cribb, 2009 FJ788496, and *Siphodera vinaledwardsii* (Linton, 1901) Linton, 1910 AY222230 (all species in the Cryptogonimidae Ward, 1917); 1942; *Schistorchis zancli* Hanson, 1953 AY222240, *Homalometron elongatum* Manter, 1947 HM038039, *Homalometron pallidum* Stafford, 1904 HM038043, *Homalometron armatum* (MacCallum, 1895) Manter, 1947 HM038045, *Homalometron synagris* (Yamaguti, 1953) Cribb and Bray, 1999 AY222243, *Neoapocreadium splendens* Cribb and Bray, 1999 AY222242, and *Callohelmins pichelinae* Cribb and Bray, 1999 FJ88495 (all species in the Apocreadiidae); *Atractotrema sigani* Durio and Manter, 1969 AY222267 (Atracotrematidae Yamaguti, 1939); *Hapladena nasonis* Yamaguti, 1970 AY222265, *Haploporus benedeni* (Stossich, 1887) Looss, 1902 FJ211237, and *Intromugil mugilicolus* (Shireman, 1964) Overstreet and Curran, 2005 KC430096 (all species in the Haploporidae Nicoll, 1914); *Robphildollfusium fractum* Paggi and Orecchia, 1963 FJ788505 and *Petalocotyle adenometra* Hall and Cribb, 2000 FJ788504 (both species in the Gyliauchenidae Fukui, 1929); *Enenterum aureum* Linton, 1910 AY222232 and *Koseiria xishaense* Gu and Shen, 1983 AY222233 (both species in the Enenteridae Yamaguti, 1958); *Gorgocephalus kyphosi* Manter, 1966 222234 (Gorgocephalidae Manter, 1966); *Preptetos caballeroi* Pritchard, 1960 AY222236, *Bianium spongiosum* Bray and Cribb, 1998 FJ788469, *Opechona kahawai* Bray and Cribb, 2003 FJ788491, and *Clavogalea trachinoti* (Fischthal & Thomas, 1968) Bray and Gibson, 1990 FJ788471

(all species in the Lepocreadiidae); *Bulbocirrus aulostomi* Yamaguti, 1933 FJ88470, *Intusatrium robustum* Durio and Manter, 1968 FJ788481, and *Myzoxenus insolens* (Crowcroft, 1945) Manter, 1947 FJ788486 (all species in the Lepidapedidae Yamaguti, 1958); *Aephnidiogenes major* Yamaguti, 1933 FJ788468 and *Neolepocreadium cabelleroi* Thomas, 1960 FJ788488 (both species in the Aephnidiogenidae Yamaguti, 1934).

For sequences used in building phylogenetic trees, the new sequences and those from GenBank were initially aligned using ClustalX 2.1 (Larkin et al., 2007) with default settings and penalties as gap opening = 10, gap extension = 0.2, delay divergent sequences = 30%, and DNA transition weight = 0.5. The alignment was adjusted by eye using Bioedit, ver. 7.1.3.0 (Hall, 1999). The alignment was further refined by eye in Bioedit and trimmed to the shortest sequence on both ends. The alignment included a total of 1,279 sites of which 1,219 could be aligned unambiguously. Regions that could not be aligned unambiguously were excluded from the analysis. Pairwise sequence comparisons were calculated from alignments of only the two sequences compared for the region in question (i.e., I did not use the manipulated alignment utilized in tree building to compute pairwise differences for species sequence comparisons).

Phylogenetic analysis of the data was performed using Bayesian Inference (BI) with MrBayes 3.1.2 software (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, ,2003). Only a single outgroup (*A. sigani*) was used in the analysis due to the requirement of the MrBayes software. The best nucleotide substitution model was estimated with jModeltest Version 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) as general time reversible with estimates of invariant sites and gamma-distributed among site-rate

variation (GTR + I + Γ) from a pool of candidate models using AIC (Akaike, 1973). The following model parameters were used in MrBayes: nst = *6*, rates = *invgamma*, ngen = *1,000,000*, and samplefreq = *100*. Posterior probabilities were approximated over 1,000,000 generations, log-likelihood scores plotted, and only the final 75% of trees was used to produce the consensus trees by setting burnin parameter to 250,000 generations with all other settings left as default.

Results

Haintestinum n. g.

Diagnosis: Body of adult elongate, approximately 5 times longer than wide, with posterior end tapered. Tegument spined, with numerous small papillae covering surface. Oral sucker relatively large, infundibuliform-shaped. Pharynx lamellar flaps comprising anterior margin fitting against posterior end of oral sucker; prepharynx indistinct. Oesophagus present. Intestine comprising paired caeca directed anteriorly and posteriorly, H-shaped; posterior arms each with anus. Testes 2, intercaecal. Pars prostatica without membrane surrounding it or terminal genitalia. Cirrus-sac absent. Ovary pretesticular. Seminal receptacle canicular. Vitellarium follicular, commencing in region of oral sucker, extending to posterior end of body. Mehlis' gland relatively large, anterioventral to anterior testis. Laurer's canal with pore dorsal. Excretory vesicle I-shaped in adult. In intestine of ostraciid fish.

Etymology: Haintestinum refers to H-shaped intestine, formed from the Latin letter H, "ha," and the Latin nominative neuter *intestinum*.

Type species: *Haintestinum amplum* n. sp.

Remarks

The new genus resembles members of the Megaperinae and members of the Apocreadiidae (subfamily Schistorchinae) in lacking a cirrus sac and having a spined tegument, dispersed eye-spot pigment, oral sucker larger than ventral sucker, pars prostatica with distinct prostatic cells free in parenchyma, extensive follicular vitellarium, intestine opening by separate ani, and an I-shaped excretory vesicle. Features possessed by *Haintestinum* n. g. that separate it from the schistorchines are a pharynx with a lobed anterior margin and an unspecialized oral sucker without a U-shaped sphincter encircling half of the oral aperture. Unusual features for Megaperidae possessed by *Haintestinum* n. g. include an oesophagus and the testis being post-ovarian and tandem-to-oblique rather than being opposite. Although *Haintestinum* n. g. has an oesophagus, it appears to be composed of the same type of cell as that for the intestine of *Thysanopharynx* as described by Manter (1933) rather than having distinctly different cell types lining the oesophagus and intestine of most other trematode groups.

Because the new genus does not fit into the most recent diagnoses for the Apocreadidae (Cribb, 2005) and the Megaperidae (Bray, 2005b), amendments are necessary.

Apocreadiidae amendment to that of Cribb (2005): Pharynx with or without lamellar flaps on anterior margin. Intestine with or without extensions anterior to bifurcation. Testis preovarian or postovarian in forebody or hindbody.

Megaperinae amendment to that of Bray (2005b): Oral sucker globular to funnel-shaped. Oesophagus present or absent. Caeca broad to narrow, with or without anterior extensions to oral sucker. Testes 2, large, opposite or tandem, in forebody or hindbody,

preovarian or postovarian. Eggs few to many. Excretory vesicle Y-shaped in cercaria, I-shaped in adults.

Haintestinum amplum n. sp.

Type host: *Acanthostracion quadricornis* (Linnaeus, 1766),

Site of infection: Intestine.

Type locality: Gulf of Mexico off Florida, (26° 57' N 83° 46'W) depth 57 m.

Other locality: Gulf of Mexico off Florida, (27° 58' N 84° 16' W) depth 60 m.

Specimens deposited: Holotype: USNPC 107290.00, Paratypes: USNPC 107291.00, 107293.00, Hologenophore: USNPC 107292.00, DNA sequence: partial 18S, entire ITS region, partial 28S: GenBank accession no. KF33447.

Etymology: The Latin, adjectival, neuter name *amplum* refers to the ample or relatively large size of this species.

Description: (Figures 1-9)

Based on 4 wholemounts, 2 hologenophores, 1 of which was used for SEM with posterior end mounted to examine ani, measurements are for 4 wholemounts and 1 partial hologenophore specimen. Body 11.9-13.4 mm long, 2.1-2.8 mm wide, with body width (BW) 18-22% of body length (BL). Tegument with small blunt spines, with posterior half of body mostly devoid of spines (Figures 6-7, 9); papillae larger than spines, located irregularly, numerous (Figures 5, 7, 9). Eye-spot pigment remnants in forebody. Oral sucker subterminal, 1,228-1,650 long, 1,101-1,350 wide, widest in anterior half, tapering posteriorly, with width 79-90% of length, with tegument extending 24-65 anterior to oral sucker, with sucker rim sharp (Figure 7). Ventral sucker slightly protruding (Figures 5-6, 8), 448-526 long, 459-515 wide, with elevated surface surrounding, cavity lacking spines

and having numerous larger papillae (Figure 8), with intrasucker distance 1,504-1,855 representing 11-13% of BL; ventral sucker length 32-36% of oral sucker length; ventral sucker width 36-43% of oral sucker width. Forebody 2,858-3,540 long representing 23-25% of BL; hindbody 8,453-9,285 representing 65-71% of BL. Pharynx 488-569 long, 547-590 wide, with approximately 8 lamellar flaps fitting inside posterior-most portion of oral sucker (Figure 2); junction between oral sucker and pharynx with numerous gland cells (Figure 1). Oesophagus 374-685 long. Caeca with H-shaped junction in forebody, bifurcating 415-541 anterior to ventral sucker, with anterior extension of caeca terminating 596-767 from anterior end of body at roughly midlevel of oral sucker or 5-6% of BL, with ani at posterior end of body.

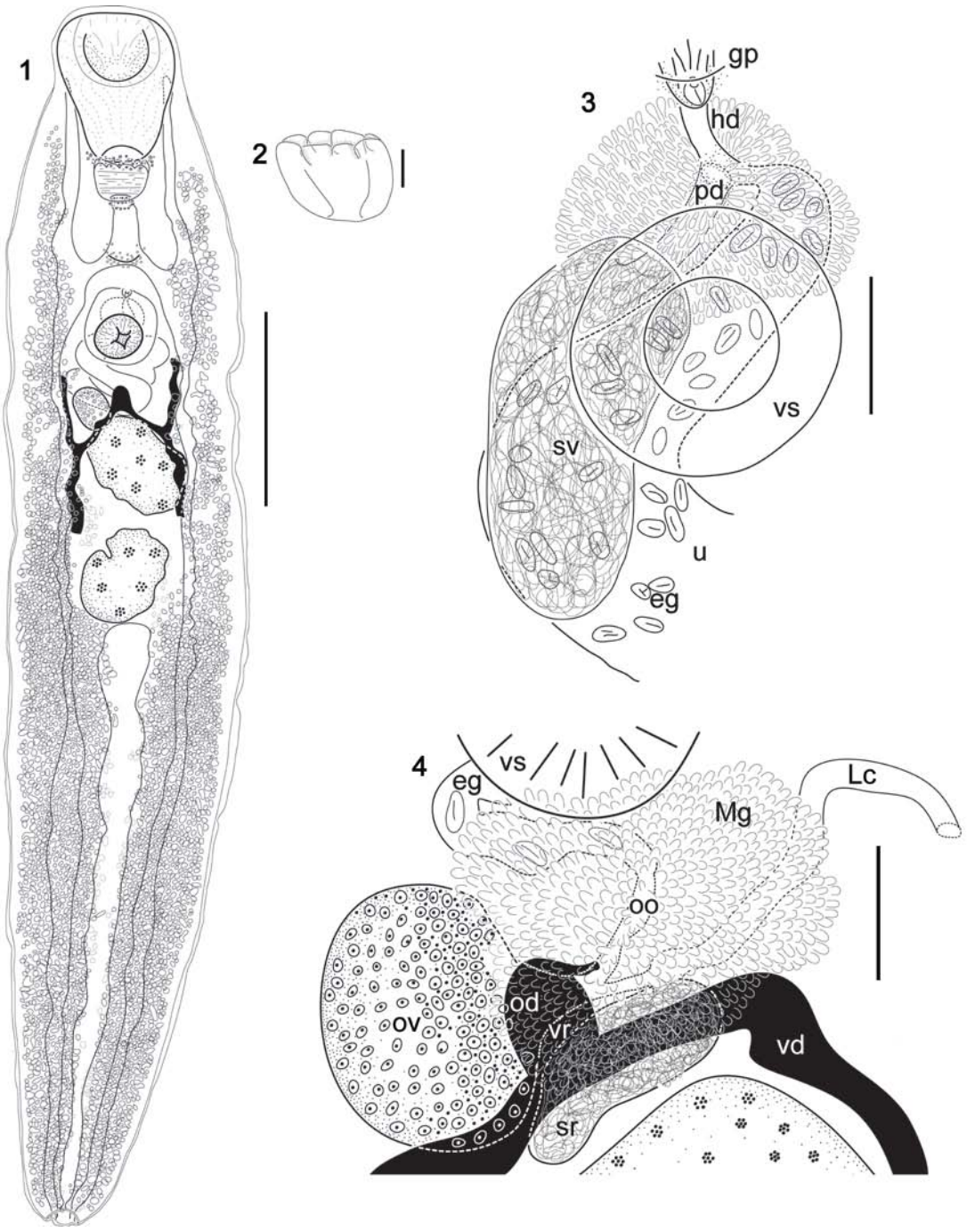
Testes tandem to slightly oblique, contiguous to separation of up to 185, with entire to irregular margins; anterior testis 652-1250 long, 535-980 wide; posterior testis 723-1190 long, 533-908 wide; testicular space 10-18% of BL; post-testicular space 5,560-8,668, representing 46-54% of BL. Seminal vesicle dextral, elongate, preovarian. Pars prostatica with prostatic glands dorsal to ventral sucker and slightly anterior, occupying area nearly as large as ventral sucker, merging with female duct as hermaphroditic duct, prostatic duct wider that hermaphroditic duct; duct nearly vertical in most specimens (making any measurement inaccurate), opening into genital atrium; genital atrium shallow, appearing laterally compressed in some specimens, immediately anterior to elevated area of ventral sucker region. Tegumental pit crescent shaped, shallow on ventral forebody, approximately half way between pharynx and ventral sucker, anterior to genital pore, surrounded by numerous gland cells.

Ovary subspherical, dextral, 465-520 long, 340-380 wide, posterior to ventral sucker, with posterior margin at level of anterior margin of anterior testis to slightly pretesticular. Seminal receptacle extending lateral to ovary, communicating with Laurer's canal; Laurer's canal muscular, opening sinistraly. Mehlis' gland medial, voluminous, mostly sinistral. Vitellarium consisting of left and right fields of follicles, larger follicles about 150 long, confluent at posterior end of body; follicles densest in testicular and post-testicular regions. Uterus originating from left side of ovary, with few loops prior to joining with prostatic duct, thin-walled, intercaecal, anterior to testis, containing hundreds of eggs, with width several eggs wide; eggs darkly tanned; distal eggs 73-83 (mean 79 n = 30) long, 28-42 (mean 34) wide, not containing developed miracidia.

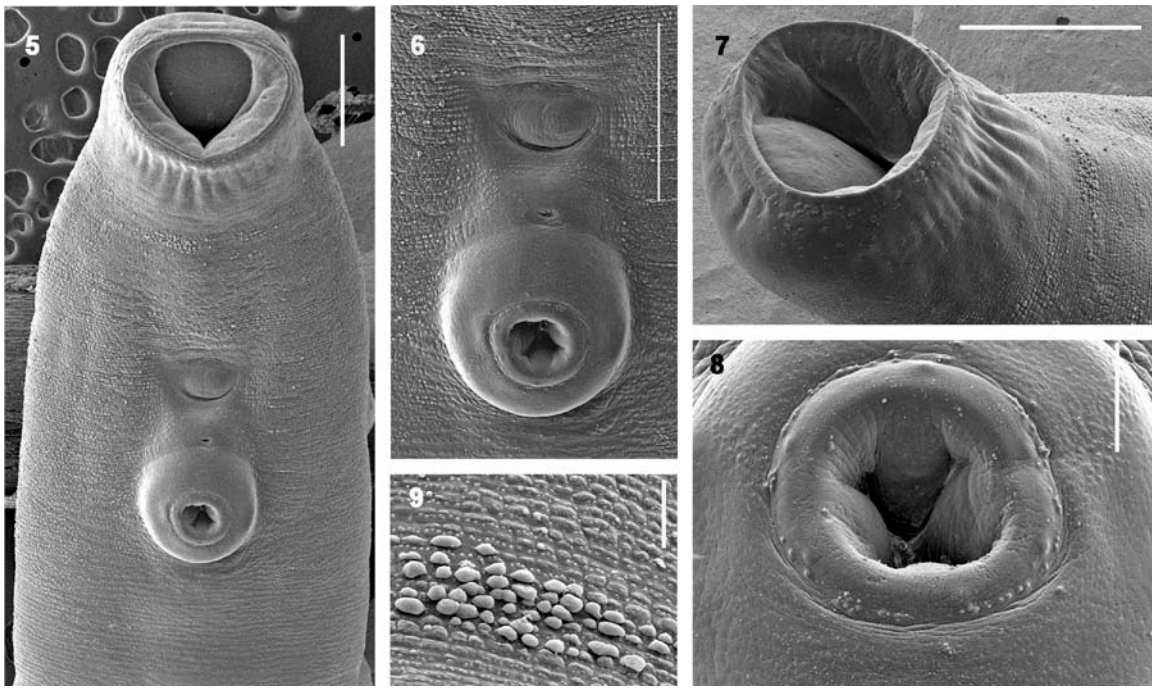
Lymphatic tubes in forebody. Excretory vesicle I-shaped, encroaching into testicular space, 5,594-6,835 long, 46-55% of BL; excretory pore terminal.

Remarks

The new species occurred in 2 of 35 scrawled cowfish, but none of the 10 examined sympatric honeycombed cowfish. Many of the morphological features exhibited are consistent with the recent diagnoses for members of the Apocreadiidae (Cribb, 2005). *Haintestinum amplum* is readily differentiated from other members of the Megaperinae by having an H-shaped intestine, testes in the hindbody, and a pretesticular ovary.



Figures VIII.1-4. Haintestinum amplum n. g., n. sp. 1. Wholemount, ventral view, holotype; 2. Pharynx, ventral view, paratype; 3. Terminal genitalia, ventral view, paratype; 4. Female complex, ventral view, amalgamation of specimens. Scale bars: 1. 2mm, 2. 200 µm 3. 500 µm, 4. 500 µm. Abbreviations: eg = egg, gp = genital pore, hd = hermaphroditic duct, Lc = Laurer's canal, Mg = Mehlis' gland, od = oviduct, oo = ootype, ov = ovary, pd = prostatic duct, sr = seminal receptacle, sv = seminal vesicle, u = uterus, vd = vitelline duct, vr = vitelline reservoir, vs = ventral sucker.



Figures VIII.5-9. SEM of *Haintestinum amplum* n. g., n. sp. 5. Anterior portion; 6. Ventral sucker, genital pore, and pit; 7. Oral sucker; 8. Area of protrusion surrounding ventral sucker; 9. Tegument, showing spines and larger apparently nonsensory papillae. Scale bars: 5. 500 µm, 6. 500 µm, 7. 500 µm, 8. 50 µm, 9. 100 µm.

Table VIII.1

Accession and deposition information for Megaperinae species collected from the Gulf of Mexico.

Species	Host	Year of collection	Depth in meters	Coordinates	GenBank	USNPC
Megapera orbicularis	*Acanthostracion quadricornis*	2010	62	24° 45' N 83° 41' W	KF33450	107287
Megapera gyrina	*A. quadricornis*	2012	1	24° 47' N 80° 56' W	KF33448	107288
Thysanopharynx elongatus	*A. quadricornis*	2008	60	27° 43' N 84° 07' W	KF33449	107289
Haintestinum amplum n.sp. type loc.	*A. quadricornis*	2010	57	26° 57' N 83° 46' W	KF33447	107290-107292
Haintestinum amplum n.sp. other loc.	*A. quadricornis*	2008	60	27° 58' N 84° 16' W		107293

Molecular sequence data

The two sequences obtained from two specimens of *H. amplum* revealed no intraspecific variation. When the new molecular sequence data for *H. amplum*, *M. gyrina*, *M. psuedura*, and *T. elongatus* were compared with available sequences of ITS2 and partial 28S from the schistorchine apocreadiid *S. zancli*, they revealed a much closer relation of *H. amplum* with the three members of the Megaperinae than with *S. zancli*, suggesting that the lobed pharynx is an important phylogenetic character uniting the species infecting tetradontiform fishes in the western Atlantic Ocean. When sequence data are considered in association with the relative position of the gonads, which was previously considered to be taxonimically important (Manter, 1933; Cable, 1954; Manter, 1963; Bray, 2005b), the position is now considered less important. *Haintestinum amplum* differed from *M. gyrina* and *M. orbicularis* by 82-92 base pairs (bp) (15.5-17.2% variable sites) in the ITS1, 40bp (13.0% variable sites) in the ITS2, and 58 bp (4.2% sequence divergence) in the 28S *Haintestinum amplum* differed from *T. elongatus* by 91bp (17.2% variable sites) in the ITS1, 35 bp (11.4% variable sites) in the ITS2, and 62 bp (4.8% sequence divergence) in the 28S; it differed from *S. zancli* by 42 bp (13.8% variable sites) in the ITS2 and 114 bp (8.8% variable sites) in the 28S. *Schistorchis zancli* differed from the megaperine species by 13.7-15.7% variable sites in the ITS2 and 8.4-8.8% for the 28S while intrageneric variation within the Megaperinae was 4.6-13.0% for the ITS2 and 2.7 to 4.5% for the partial 28S. The tree resulting from the BI analysis (1,000,000 generations) showed very high support for the Megaperinae (Figure 10). *Haintestinum amplum* formed a well-supported clade with the three recognized species of megaperines. The megaperines were most closely related to *S. zancli*, a species considered to belong to

the Apocreadiidae (Cribb & Bray, 1999; Cribb, 2005) rather than to members of the Haploporidae or Lepocreadioidea.

Discussion

Since no member of the Megaperidae was included in the recent phylogenetic analyses by Olson et al. (2003), Bray et al. (2009), or Bray and Cribb (2012) (using the phylogeny of Bray et al., 2009), the family had been retained in the Lepocreadioidea for convenience of identification, pending phylogenetic analyses (Bray, 2005b). The Megaperidae was included in the Lepocreadioidea based on morphologic similarity to the Apocreadiidae (Bray, 2005a), although Olson et al. (2003) suggested that Apocreadiidae may require its own superfamily. My sequence data and BI analysis support the monophyly of the Apocreadiidae when the Megaperinae is included. The close association of *S. zancli* with the megaperines indicates that megaperines are more closely allied with apocreadiids than with the Haploporoidae, Lepocreadioidea, or Opisthorchioidea Braun, 1901 based on taxa included here. Also, a nuceolotide BLAST search of GenBank of the 28S fragment used here for *H. amplum* and the other megaperine species resulted in the highest maximum identity score being that of *S. zancli*, followed by various other species of apocreadiids. I advocate the transfer of members of the Megaparidae as well as *H. amplum* into the family Apocreadiidae within the subfamily Megaperinae. No molecular evidence exists for members of Liliatrematidae and Deropristidae, which have a questionable status but are still considered Lepocreadioid; members of both families possess a well-developed cirrus-sac, which most likely precludes a close association of either with Apocreadiidae. The presented phylogeny and morphological observations suggest that the Megaperinae and

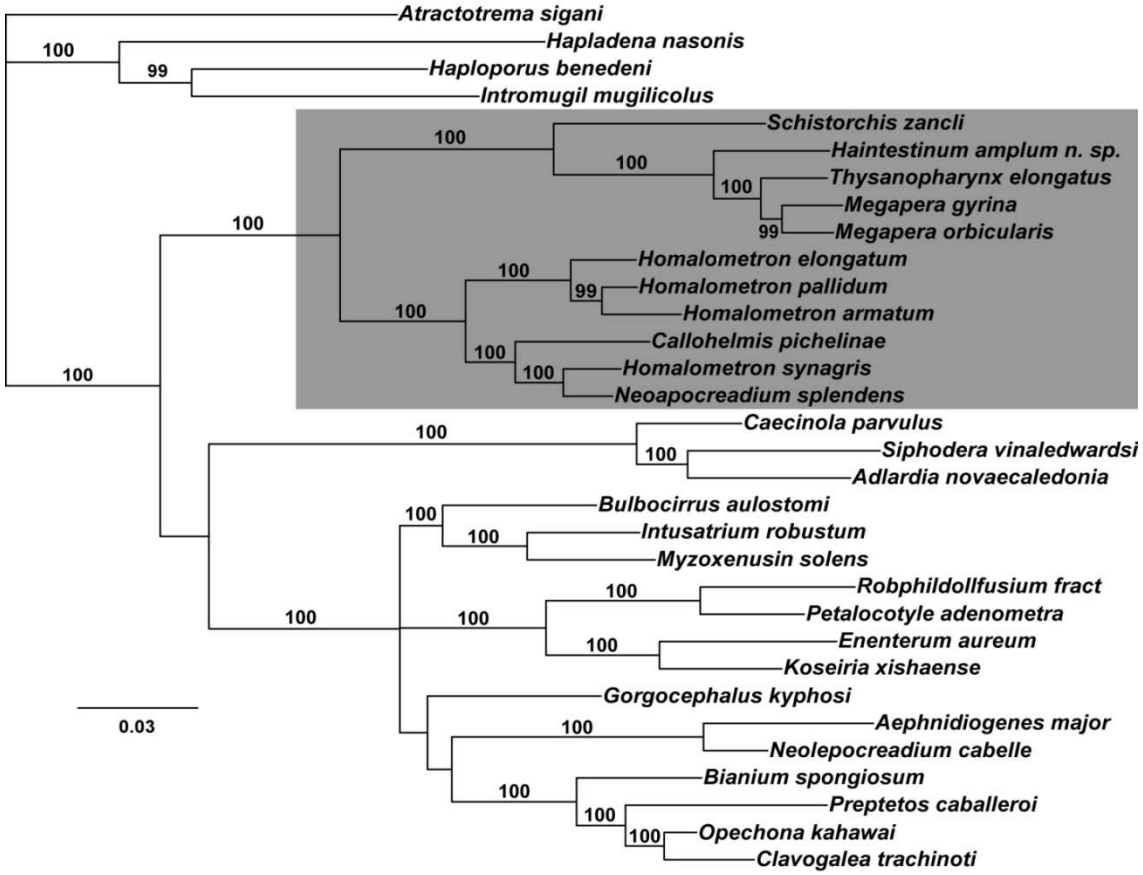


Figure VIII.10. Phylogenetic relationships among purported relatives of the Megaperinae 10. Bayesian analysis (1,000,000 generations) tree of partial sequences of 28S rDNA gene. Posterior probabilities greater than 80% are shown above internodes. Shaded rectangle indicates the Apocreadiidae that includes *Haintestinum amplum* n. g., n. sp.

Schistorchinae are very closely related. In particular, members of both groups share features such as the possession of an anus or ani, while other members do not. The megaperines can be differentiated from the schistorchiines by having a pharynx with a lobed anterior rim and lack of a U-shaped sphincter half encircling the oral aperture.

According to the Apocreadiidae key presented by Cribb (2005), *H. amplum* would key to Apocreadinae rather than Schistorchinae, to which *Haintestinum* shows the closest molecular phylogenetic affinity; however, molecular data are available for only one schistorchine, *S. zancli*. None of the known members of the Apocreadiinae or

Postporinae exhibits an H-shape intestine that terminates posteriorly in paired ani, a pharynx with anterior lamellar flaps, or the lack of a partial muscular U-shaped sphincter encircling the anterior aperture of the oral sucker. Consequently those features precluded placement of *Haintestinum* in the Schistorchinae according to Cribb (2005). Also, members of the Schistorchinae as currently recognized are restricted to the Indian and Pacific oceans rather than the Atlantic Ocean as for megaparines. I consider the presence of an anus (*Sphincterostoma*) or ani (*Megapera*, *Thysanopharynx, Haintestinum*, *Sphincteristomum, Megacreadium*, *Schistorchis*, and *Neomegacreadium* Machida and Kuramochi, 1999) to be a critical feature separating the Schistorchinae and Megaperinae from the Postporinae and Apocreadinae. The major ambiguity of the new species and the other megaperines is the position of the gonads. From a morphological point of view, the position of the gonads in *H. amplum* would strongly support placement of this species within the Schistorchinae rather than Megaperinae. However, unusual morphological features of *H. ampla* include the possession of a raised fleshy area surrounding the ventral sucker and a large crescent-shaped pit of unknown function located anterior to the raised area. A raised fleshy area of the tegument also occurs in *M. pseudura*, with a shallow pit anteriorly and ventral to the caecal bifurcation. Numerous gland cells of uncertain function lying between the ventral surface and the intestine of *H. amplum* more likely relate to the pit than to the intestine. Of the specimens of *Megapera* and *Thysanopharynx* I examined, only *M. pseudura* was conclusive for this pit; it is almost certainly present in *M. gyrina* and inferred for other species of *Megapera* and requires further investigation into its function and occurrence in these and other megaperines.

Cable (1954) reported a cercaria that he presumed was *M. gyrina* from *C. convexa* based on morphologic features. Characters that support the cercaria belonging to *Megapera* are convincing despite an absence of molecular data or demonstration of a corresponding adult; it includes eye-spots, large oral sucker, large pharynx, post-testicular ovary, and prepharynx (short, wide, and ribbed). The main discrepancy between the reported cercaria and adult megaperines is the possession of a Y-shaped excretory vesicle in the cercaria, which apparently degenerates into I-shaped in the adult. The putative cercaria of *M. gyrina* possesses a pair of notched lateral fins and a low ventral fin along most of the length of the tail but neither spines nor setae on its body. The body and tail of known apocreadines possess setae without lateral or ventral fins (Hopkins, 1937; Stunkard, 1963; Stunkard, 1964; Scholz et al., 1995;). In contrast, known life-cycles of apocreadiines involve three hosts (Stunkard, 1963; Stunkard, 1964; Scholz et al., 1995). This contrast between the cercarial stage of the Megaperinae and Apocreadinae is substantial, and, if the schistorchine cercariae are found to be similar to that for *M. gyrina*, this diagnosis might indicate that Schistorchinae and Megaperinae represent a family.

Whereas my molecular analysis shows good support for the inclusion of the megaperines within the Apocreadiidae, the placement of *H. synagris* nested between *N. splendens* and *C. pichelinae* (Figure 10) suggests that the inclusion of *H. synagris* in *Homalometron* Stafford, 1904 may need critical evaluation. Clearly, the addition to my analysis of more species of Schistorchinae is needed to clarify the interrelationships and more complete evolution of members of the Apocreadiidae.

APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICES OF COMMITTEE ACTION

The University of Southern Mississippi

Institutional Animal Care and Use Committee

118 College Drive #5147
Hattiesburg, MS 39406-0001
Tel: 601.266.6820
Fax: 601.266.5509
www.usm.edu/spa/policies/animals

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: **07092701**
PROJECT TITLE: **Collection, Maintenance, Life Histories, Taxonomy, & Experimental Studies With Parasites, Disease-Causing Agents, & Host Biology of Fishes**
PROPOSED PROJECT DATES: **2005 - 2010**
PROJECT TYPE: **Project Renewal**
PRINCIPAL INVESTIGATOR(S): **Robin M. Overstreet, Ph.D.**
COLLEGE/DIVISION: **College of Marine Science**
DEPARTMENT: **Coastal Sciences**
FUNDING AGENCY/SPONSOR: **National Science Foundation, PEET & SGER, U.S. Department of Commerce, BCARC, U.S. Department of Agriculture CSREES, U.S. Marine Shrimp Farming Program**
IACUC COMMITTEE ACTION: **Full Committee Review Approval**
EXPIRATION DATE: **September 30, 2010**

Robert C. Bateman, Jr., Ph.D.
IACUC Chair

9-27-07
Date

The University of
Southern Mississippi

Institutional Animal Care and Use Committee

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Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: **10100105**
PROJECT TITLE: **Collection, Maintenance, Life Histories, Taxonomy, and Experimental Studies with Parasites, Disease-Causing Agents, and Host Biology of Fishes**
PROPOSED PROJECT DATES: **10/01/2010 to 09/30/2013**
PROJECT TYPE: **New Project**
PRINCIPAL INVESTIGATOR(S): **Robin M. Overstreet, Ph.D.**
COLLEGE/DIVISION: **College of Science & Technology**
DEPARTMENT: **Coastal Sciences**
FUNDING AGENCY/SPONSOR: **National Science Foundation, National Marine Fisheries Service, National Oceanic & Atmospheric Administration, Departmental Awards**
IACUC COMMITTEE ACTION: **Full Committee Review Approval**
PROTOCOL EXPIRATION DATE: **09/30/2013**

Robert C. Bateman, Jr., Ph.D.
IACUC Chair

9-14-2010
Date

REFERENCES

Ahmad, J. (1987). On seven new digenetic trematodes of marine fishes from the arabian sea, off the Panjim coast, Goa. *Pakistan Journal of Zoology, 19,* 321-340.

Akaike, H. (1973). Information theory as an extension of the maximum likelihood principle. *In* Second international symposium on information theory. *eds*. Petrov, B. N. & Csaki, F. Budapest, Hungary: Akademiai Kiado, pp. 267-81.

Al-Daraji, S. A. M. (1999). *Carassotrema lizae* sp. nov. (Trematoda: Haploporidae) from the gut of the mugilid fish, *Liza macrolepis* (Smith, 1849). *Marina Mesopotamica*, *14*, 83-89.

Belous, E. V. (1954). [Contribution to the systematics of the trematode family Haploporidae Nicoll, 1914] *Trudy Gel'mintologicheskoi Laboratorii, Akademiya Nauk SSR, 7*, 277-279. (In Russian).

Bilqees, F. M. (1980). Three trematodes including two new species from fishes of the Karachi Coast. *Zoologica Scripta*, *9*, 89-91.

Bilqees, F. M. (1981). Digenetic trematodes fishes of Karachi Coast. Karachi, Pakistan: Kifayat Academy, 207 pp.

Blasco-Costa, I., Balbuena, J. A., Kostadinova, A., & Olson, P. D. (2009). Interrelationships of the Haploporinae (Digenea: Haploporidae): a molecular test of the taxonomic framework based on morphology. *Parasitology International, 58*, 265-269.

Blasco-Costa I., Balbuena J. A., Kostadinova A., & Olson, P. D. (2010). Molecules and Morphology reveal cryptic variation among digeneans infecting sympatric mullets in the Mediterranean. *Parasitology, 137*, 287-302.

Blasco-Costa, I., Gibson, D. I., Balbuena, J. A., Raga, J. A., & Kostadinova, A. (2009). A revision of the Haploporinae Nicoll, 1914 (Digenea: Haploporidae) from mullets (Mugilidae): *Haploporus* Looss, 1902 and *Lecithobotrys* Loos, 1902. *Systematic Parasitology, 72*, 107-133.

Blasco-Costa, I., Montero, F. E., Gibson, D. I., Balbuena, J. A., & Kostadinova, A. (2009). A revision of the Haploporinae Nicoll, 1914 (Digenea: Haploporidae) from mullets (Mugilidae): two new haploporine genera and a key to the genera of the subfamily. *Systematic Parasitology, 72*, 207-215.

Blasco-Costa, I., Montero, F. E., Gibson, D. I., Balbuena, J. A., Raga, J. A., & Kostadinova, A. (2009). A revision of the Haploporinae Nicoll, 1914 (Digenea: Haploporidae) from mullets (Mugilidae): *Saccocoelium* Looss, 1902. *Systematic Parasitology, 72*, 159-186.

Bray, R. A. (2005a). Superfamily Lepocreadioidea Odhner, 1905. *In:* Jones A, Bray RA, Gibson DI, (Eds) *Keys to the Trematoda*, Vol. 2, Wallingford, UK: CABI International, Museum, pp. 541-543.

Bray, R. A. (2005b). Family Megaperidae Manter, 1934. *In:* Jones A, Bray RA, Gibson DI, (Eds) *Keys to the Trematoda*, Vol. 2, Wallingford, UK: CABI International, Museum, pp. 683-685.

Bray, R. A. (2009). Waeschenbach A, Cribb TH, Weedall GD, Dyal P, Littlewood DTL. The phylogeny of the Lepocreadioidea (Platyhelminthes, Digenea) inferred from nuclear and mitochondrial genes: Implication for their systematics and evolution. *Acta Parasitologica, 54*, 310-329.

Bray, R. A., & Cribb T. H. (2012). Reorganisation of the superfamily Lepocreadioidea Odhner, 1905 based on an inferred molecular phylogeny. *Systematic Parasitology, 83*, 169-177.

Brooks, D. R., O'Grady T. O., & Glen D. R. (1985). Phylogenetic analysis of the Digenea (Platyhelminthes: Ceromeria) with comments on their adaptive radiation. *Canadian Journal of Zoology, 63*, 411-443.

Bykhovskaya-Pavlovskaya, I. E., & Kulakova A. P. (1987). Classes Aspidogastrea Faust and Tang, 1936 and Trematoda Rudolphi, 1808. *In:* Bauer O.N. (Ed) *Key to the parasites of freshwater fishes of the USSR fauna, Parasitic metazoans, Pt. 2.*Vol. 3, St. Petersburg, RU. Publishing House Nauka, Leningrad, pp. 76-198 (in Russian).

Cable, R. M. (1954). Studies on marine digenetic trematodes of Puerto Rico. The life cycle in the family Megaperidae. *Journal of Parasitology, 40*, 202-208.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution, 17*, 540-552.

Conroy, G., & Conroy, D. A. (1984). Diseases and parasites detected in the grey mullets (Mugilidae) from the coastal waters of Sao Paulo State, Brasil. I. Adult silver mullet (*Mugil curema* Val., 1836). *Rivista Italiana di Piscicultura e Ittiopatologia 19*, 14-28.

Conroy, G., & Conroy, D. A. (1986). First records of some digenetic trematodes occurring as intestinal parasites of the silver mullet (*Mugil curema* Val., 1836). *Rivista Italiana di Piscicultura e Ittiopatologia, 21*, 100-106.

Cribb, T. H. (2005). Family Apocreadiidae Skrjabin, 1942. *In:* Jones A, Bray RA, Gibson DI, (Eds) *Keys to the Trematoda,* Vol. 2, Wallingford, UK: CABI International, Museum, pp. 621-639.

Cribb, T. H., & Bray R. A. (1999). A review of the Apocreadiidae Skrjabin, 1942 (Trematoda: Digenea) and description of Australian species. *Systematic Parasitology, 44,* 1-36.

Cribb, T. H., & Bray, R. A. (2010). Gut wash, body soak, blender and heat-fixation: approaches to effective collection, fixation and preservation of trematodes of fishes. *Systematic Parasitology, 76,* 1-7.

Datta, I.B., Manna, B., & Kundu, D.K. (1997). On a new species of the genus Unisaccus Martin, 1973 (Trematoda: Haplopodidae [sic]) from Digha Coast, Bay of Bengal. *Proceedings of the Indian Science Congress Association, 84,* 92-93.

Diaz, M. T., Bashirullah, A. K., Hernandez, L. E., & Gomez, E. (2009). Life cycle of *Culuwiya tilapiae* (Nasir and Gomez, 1976) (Trematoda: Haploporidae) in Venezuela. *Revista Cientifica, 5*, 439-446.

Durand, J. D., Chen, W. J., Shen, K. N., Fu, C., & Borsa, P. (2012). Genus-level taxonomic changes implied by the mitochondrial phylogeny of grey mullets (Teleostei: Mugilidae). *Comptes Rendus Biologies, 335*, 687-697.

Durand, J. D., Shen, K. N., Chen, W. J., Jamandre B. W., Blel, H., Diop, K., Nirchio, M., Garcia de Leon, F. J., Whitfield, A. K., Chang, C. W., & Borsa, P. (2012). Systematics of the grey mullet (Teleostei: Mugiliformes: Mugilidae): molecular phylogenetic evidence challenges two centuries of morphology-based taxonomy. *Molecular Phylogenetics and Evolution, 64*, 73-92.

Ferandes, B. M. M., & Cohen, S. C. (2006). A new species of *Intromugil* Overstreet and Curran, 2005 (Digenea, Haploporidae) from the Brazilian marine fish *Mugil liza* Valenciennes (Perciformes, Mugilidae). *Zootaxa, 1328*, 63-68.

Froese, R., & Pauly, D. (Eds) (2013). *FishBase. World Wide Web electronic publication.* http://www.fishbase.org, version (12/2013).

Guindon, S., & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology, 52*, 696-704.

Gupta, N. K., & Miglani, A. (1976). Digenetic trematodes from marine fish food fishes and wild ducks of Port Blair (Andaman and Nicobar islands) India. *Revista Iberica de Parasitologia, 36,* 219-248.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Research*, *41*, 95-98.

Harrison, I. J., & Senou, H. (1997). Order Mugiliformes. Mugilidae. Mullets. *In:* Carpenter, K. E., & Niem, V. H. (Eds) *FAO species identification guide for fishery purposes. The living marine resources of the Western Central Pacific. Volume 4. Bony fishes part 2 (Mugilidae to Carangidae)*. Rome, IT: Food and Agricultural Organization of the United Nations, pp. 2069-2108.

Hassanine, R.M.E-S., & Ahmed, A. (2002). Endoparasitic helminthes of the striped mullet, Mugil cephalus, from the northern Red Sea: Effect of the host's mode of life on the incidence and intensity of infection. *Egyptian Journal of Aquatic Biology and Fish, 6*, 41-58.

Hopkins S. H. (1937). A new type of allocreadiid cercaria: the cercaria of *Anallocreadium* and *Microcreadium*. *Journal of Parasitology*, *23*, 94-97.

Huelsenbeck, J. P., & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogeny. *Bioinformatics, 17,* 754-755.

Huelsenbeck, J. P., Ronquist, F., Nielsen, R., & Bollback, J. P. (2001). Bayesian inference of phylogeny and its impact on evolutionary biology. *Science, 294*, 2310-2314.

Hutton, R. F. (1964). A second list of parasites from marine and coastal animals of Florida. *Transactions of the American Microscopical Society 84*, 439-447.

Hutton, R. F., & Sogandares-Bernal, F. (1964). A list of parasites from marine and coastal animals of Florida. *Transactions of the American Microscopical Society, 79*, 287-292.

Keller, A., Schleicher, T., Schultz, J., Müller, T., Dandekar, T., & Wolf, M. (2009). 5.8S-28S rRNA interaction and HMM-based ITS2 annotation. *Gene, 430*, 50-57.

Kohn, A., Fernandea, B. M. M., & Gibson, D.I. (1999). *Chalcinotrema thatcheri* n. sp. (Digenea: Haploporidae) from Brazilian freshwater fishes, a redescription of *C. ruedasueltensis* Thatcher, 1978 and comments on the validity of the genus. *Systematic Parasitology, 44*, 211-215.

Kottelat, M. (2006). Fishes of Mongolia. A check-list of the fishes known to occur in Mongolia with comments on systematics and nomenclature. Washington DC, USA: The World Bank, pp. 103.

Kulakova, A. P., & Ha Ky P. (1976). *Carassotrema koreanum* and a new species of *Carassotrema* (Trematoda, Waretrematidae) from freshwater fish in North Vietnam. *Parazitologiya 10*, 460-462 (in Russian).

Larkin M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam H., Valentin, F., Wallace I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., & Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics, 23*, 2947-2948.

Li, M. M. (1984). Parasites of the mullets Mugil cephalus (Linnaeus) and Liza haematocheila (Temminck et Schlegel) in the areas of Bohai Gulf. I. Hangu area. *Acta Zoologica Sinica, 30*, 153–158. (In Chinese).

Liu, S. (2002). One new species of Saccocoelioides (Digenea: Haploporidae) from Liza carinatus in Taiwan Strait. *Journal of Oceanography in Taiwan Strait, 21*, 37-44 (in Chinese with English translation).

Liu, S.F. (2003). A new species of *Carassotrema* Park, 1938 (Digenea: Waretrematidae). *Acta Hydobiologica Sinca, 27*, 283-285.

Liu, S., & Yang T. (2002). Two new species of *Pseudohapladena* Yamaguti, 1952 (Digenea: Waretrematidae) from mugilid fish from the Taiwan Straits, China. *Journal of Parasitology. 88*, 358-361.

Liu, S., & Yang, T. (2003). A redescription and a new geographical record of *Waretrema piscicola* Srivastava, 1939 (Trematoda: Waretrematidae) in South China Sea. *Journal of Tropical Oceanography, 22*, 82-86.

Long, S., & Lee W.C. (1958). Parasitic worms from Tai Hu fishes: Digenetic trematodes. II. Opisthorchiidae and other families, with a description of a new species of *Opisthorchis*. *Acta Zoologica Sinica, 10*, 369-376.

Machida, M. (1996). Digenean trematodes from mullets in Japanese waters. *Japanese Journal of Parasitology, 45*, 123-133.

Manter, H. W. (1933). A new family of trematodes from marine fishes. Transactions of the *American Microscopical Society, 52*, 233-242.

Manter, H. W. (1963). Studies on digenetic trematodes of fishes of Fiji. II. Families Lepocreadiidae, Opistholebetidae, and Opecoelidae. *Journal of Parasitology, 49*, 99-113.

Martin, W. E. (1973a). Life history of *Saccocoelioides pearsoni* n. sp. and the description of *Lecithobotrys sprenti* n. sp. (Trematoda: Haploporidae). *Transactions of the American Microscopical Society, 92*, 80-95.

Martin, W. E. (1973b). A new subfamily, two new genera, and three new species of haploporid trematodes. *Proceeding of the Helminthological Society of Washington. 40*, 237-240.

Martin, W. E. (1973c). A new genus and species of haploporid trematode (Haploporidae: Trematoda) from Australian mullet. *Bulletin of the Southern California Academy of Sciences 72*, 166-168.

Mehra, H. R. (1961). New superfamily Haploporoidea (suborder Echinostomata Szidat, 1939; order Echinostomida La Rue, 1957 syn. Fasciolatoidea Szidat, 1936). *Proceeding of the National Academy of Sciences, India Section, B 31*, 383-384.

Moravec, F., & Sey, O. (1989). Some trematodes of freshwater fishes from north Vietnam with a list of recorded endohelminthes by fish host. *Folia Parasitologica. 36*, 243-262.

Nagaty, H. F. (1948). Trematodes of the Red Sea. Part 4. On some new forms with a single testis. *Journal of Parasitology, 34*, 355-363.

Nasir, P., & Gómez, Y. (1976). *Carassotrema tilapiae* n. sp. (Haploporidae Nicoll, 1914) from the freshwater fish, *Tilapia mossambica* (Peters), in Venesula. *Rivista di Parassitologia 37*, 207-228.

Nolan, M. J., & Cribb, T. H. (2005). The use and implications of ribosomal DNA sequencing for the discrimination of digenean species. *Advances in Parasitology, 60*, 101-163.

Olson P. D., Cribb, T. H., Tkach, V. V., Bray, R. A., & Littlewood, D. T. J. (2003). Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *International Journal for Parasitology, 33*, 733-55.

Olson, P. D., & Tkach, V. V. (2005). Advances and trends in the molecular systematics of the parasitic Platyhelminthes. *Advances in Parasitology, 60*, 165-243.

Overstreet, R. M. (1971). Some adult digenetic trematodes in striped mullet from the Northern Gulf of Mexico. *Journal of Parasitology, 57*, 967-974.

Overstreet, R. M. (2007). Effects of a hurricane on fish parasites. *Parassitologia, 49*, 161-168.

Overstreet, R. M., & Curran, S. S. (2005). Family Haploporidae Nicoll, 1914. *In*: Jones, A., Bray, R. A., & Gibson, D. I. (Eds) *Keys to the Trematoda*. Vol.2. Wallingford, UK: CAB International, pp. 129-167.

Park, J. T. (1938). A new fish trematode with single testis from Korea. *Keijo Journal of Medicine 9*, 290–298.

Parker, J. H., Curran, S. S., Overstreet, R. M., & Tkach, V. V. (2010). Examination of *Homalometron elongatum* Manter, 1947 and description of a new congener from

Eucinostomus currani Zahuranec, 1980 in the Pacific Ocean off Costa Rica. *Comparative Parasitology, 77,* 154-163.

Pleijel, F., Jondelius, U., Norlinder, E., Nygren, A., Oxelman, B., Schander, C., Sundberg, P., & Thollesson, M. (2008). Phylogenies without roots? A plea for the use of vouchers in molecular phylogenetic studies. *Molecular Phylogenetics and Evolution, 48,* 369-371.

Posada, D. (2008). jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution, 25,* 1253-1256.

Pulis, E. P., & Overstreet, R. M. (2013). Review of haploporid (Trematode) genera with ornate muscularisation in the region of the oral sucker, including four new species and a new genus. *Systematic Parasitology. 84,* 167-191.

Pulis, E. P., Fayton, T. J., Curran, S. S., & Overstreet, R. M. (2013). A new species of *Intromugil* (Digenea: Haploporidae) and redescription of *Intromugil mugilicolus*. *Journal of Parasitology, 99,* 501-508.

Rekharani, Z., & Madhavi, R. (1985). Digenetic trematodes from mullets of Visakhapatnam (India). *Journal of Natural History, 19,* 929-951.

Ronquist, F., & Huelsenbeck, J. P. (2003). MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics, 19,* 1572-1574.

Scholz, T., Pech-Ek, M. C. F., & Rodriguez-Canul, R. (1995). Biology of *Crassicutis cichlasomae*, a parasite of cichlid fishes in Mexico and Central America. *Journal of Helminthology, 69,* 69-75.

Shameem, U., & Madhavi, R. (1991). Observations on the life-cycles of two haploporid trematodes, *Carassotrema bengalense* Rekharani & Madhavi, 1985 and *Saccocoelioides martini* Madhavi, 1979. *Systematic Parasitology*, *20*, 97-107.

Sheena P., & Janardanan, K. P. (2007). The life cycle of *Hapladena gymnocephali* sp. nov. (Digenea: Haploporidae) from the bald glassy perchlet *Ambassis gymnocephalus* in Kerala, India. *Journal of Helminthology, 81,* 301-306.

Shen, J. W., & Qiu, Z. Z. (1995). Studies on the trematodes of fishes from the Yellow Sea and the Bo Hai Sea. Beijing: Science Press, 207 pp. (In Chinese).

Shireman, J. V. (1964). *Carassotrema mugilicola*, a new haploporid trematode from the striped mullet, *Mugil cephalus*, in Louisiana. *Journal of Parasitology*, *50,* 555-556.

Skinner, R. (1975). Parasites of the striped mullet, *Mugil cephalus*, from Biscayne Bay, Florida, with descriptions of a new genus and three new species of trematodes. *Bulletin of Marine Science*, *25*, 318-345.

Sogandares-Bernal, F., & Hutton, R. F. (1959). Studies on helminth parasites from the coast of Florida. I. Digenetic trematodes of marine fishes from Tampa and Boca Ciega bays with descriptions of two new species. *Bulletin of Marine Science of the Gulf and Caribbean, 9*, 53-68.

Srivastava, H. D. (1937). The morphology and systematic relationships of a new parasite-*Waretrema piscicola* - gen., et sp., nov., referable to a new family - Waretrematidae - n. fam., of digenetic trematodes. *Proceedings of the 24th Indian Science Congress (Hyderabad) 1937*, pp, 399-400.

Srivastava, H. D. (1939). The morphology and systematic relationships of a new parasite - *Waretrema piscicola* - gen. et. sp. nov., referable to a new family - Waretrematidae - of digenetic trematodes. *Indian Journal of Veterinary Science and Animal Husbandry*, 9, 169-174.

Stunkard, H. W. (1963). Succesive stages in the life-cycle of *Homalometron pallidum*, a digenetic trematode from the intestine of *Fundulus heteroclitus*. *Biological Bulletin, 125*, 393.

Stunkard, H. W. (1964). The morphology, life-history, and systematics of the digenetic trematode, *Homalometron pallidum* Stafford, 1904. *Biological Bulletin, 126*, 163-173.

Talavera, G., & Castresana, J. (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology, 56*, 564-577.

Tang, Z. Z., & Lin, H. M. (1963). in Chinese Zoological Congress, Peking, 1963 (in Chinese).

Tang, Z., & Lin, H. (1979). Studies on *Carassotrema* Park, 1938 life-histories and distribution. *Journal of Xiamen University, 1*, 81-98 (In Chinese with English Summary).

Tkach, V. V., & Kinsella, J. M. (2011). New *Macroderoides* (Digenea: Macroderoididdae) from Florida gar, with molecular phylogeny of the genus. *Journal of Parasitology, 97*, 920-923.

Tkach, V. V., Littlewood, D. T. J., Olson, P. D., Kinsella, J. M., and Swiderski, Z. (2003). Molecular phylogenetic analysis of the Microphalloidea Ward, 1901 (Trematoda: Digenea). *Systematic Parasitology*, *56*, 1–15.

Tkach, V. V., & Mills, A. M. (2011). *Alloglossidium fonti* sp. nov. (Digenea, Macroderoididae) from black bullheads in Minnesota with molecular differentiation from congeners and resurrection of *Alloglossidium kenti*. *Acta Parasitologica*, *56*, 154-162.

Tkach, V. V., Pulis, E. E., & Overstreet, R. M. (2010). A new *Paramacroderoides* (Digenea: Macroderoididae) from two species of gar in the southeastern United States. *Journal of Parasitology*, *96,* 1002-1006.

Travassos L., Freitas, J. F. T., & Bührnheim, P. F. (1965). Trematodeos de peixes do litoral capixaba: *Chalcinotrema simonei* sp. n., parasita de tainha. *Atlas da Sociedade de Biologia do Rio de Janeiro*, *9*, 38-40 (in Portuguese).

Velasquez, C. C. (1961). Some digenetic trematodes of Philippine food fishes. *Journal of Parasitology*, *47*, 521-526.

Wang, S. (1991). Haploporidae. *In:* Wu, B., Sun, X., & Song, C. (Eds) *Fauna of Zhejiang Trematoda*. Haungzhou, CN: Zhejiang Science and Technology Publishing House, pp. 295-301. (in Chinese).

Cribb, T. H. (2005). Family Apocreadiidae Skrjabin, 1942. *In:* Jones A, Bray RA, Gibson DI, (Eds) *Keys to the Trematoda*, Vol. 2, Wallingford, UK: CABI International, Museum, pp. 621-639.

Wang, W.-j. (1973). Megasolenidae. *In*: An illustrated guide to fish diseases and causative pathogenis fauna and flora in the Hubei province. (Ed) Institute of Hydrobiology) Academia Sinica Press, Bejing, p. 183-185. (In Chinese).

Wang, W,-j., & Pan, J.-p. (1984). Studies on the genus *Carassotrema* Park, 1938 (Digenea: Haploporidae Nicoll, 1914) in China. *In*: Institute of Hydrobiology Academia Sinica (Ed.) Parasitic organisms of freshwater fish of China. Beijing: Agricultural Publishing House, pp. 139-146. (In Chinese.).

Wang, P., & Jiang, X. (1985). Studies on the helminth fauna of Sichuan Part 1. Trematodes of fishes. *Sichuan Journal of Zoology. 4*, 1-7 (In Chinese with English summary).

Wang, P-Q., Zhao, Y-R., Chen, Q-G., & Tao, J-Y. (1983). Notes on some species of parasitic helminths from freshwater fishes and five new species in Hongze Lake. *Journal of Fujian Teacher's University Natural Science Edition. 1983*, 125–134. (In Chinese).

Wang, X. Y., & Zhou, J. Y. 1993. Fauna of Jiangxi: Trematodes of animals and man. Jiagxi Science and Technology Press, China p. 530-539 (In Chinese).

Wu B. H., Sun, X., & Song C. C. (1991) Fauna of Zhejiang (Trematoda). Hang Zhou: Zhejiang Science and Technology Publishing House, 416 pp. (In Chinese).

Yamaguti, S. (1971). Synopsis of digenetic trematodes of vertebrates. Parts 1& 2. Keigaku Publishing Company. Tokyo, Japan. 1074 pp.

Yu, S. Z., Peng, W. F., & Liu, S. F. (2005). *Carassotrema flecterotestis* (Zhukov, 1917) comb. nov. and observation of its miracidia development in vitro. *Journal of Oceanography in Taiwan Strait. 24*, 97-103. (In Chinese).

Zhukov, E. V. (1971). New trematodes of marine and freshwater fishes from the basins of the Japan and Yellow Seas. *Parazitologiya. 5*, 155-161. (In Russian).

Zhukov, E. V. (1972). New genera of trematodes from marine fishes of India. *Parazitologiya 6*, 346-350. (In Russian.).